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(71) Applicant (*for all designated States except US*): OS-COTEC INC. [KR/KR]; 2-17 Ohmok-ri, Sungkeo-up, 330-831 Cheonan-si, Chungcheongnam-do (KR).

(71) Applicants and
(72) Inventors: **RYOO, Hyun-Mo** [KR/KR]; #102-1107 Jang-won-mantion, 330 Bumou 4-dong, Soosung-ku, 706-014 Daeku-si (KR). **KIM, Jung-Keun** [KR/KR]; #508-201, Hyojachon Samhwan Apt., Seohyun-dong, Bundang-ku, 463-050 Seongnam-si, Kyeongki-do (KR).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): **KIM, Hyun-Jung** [KR/KR]; #B-501 Samik-Villa, 285-23 Shinam 4-dong, Dong-ku, 701-816 Daeku-si (KR). **KIM, Jung-Hwan** [KR/KR]; #101-201 Chowon Family Town, 112-2 koe-jeon-dong, dong-ku, 701-320 Daeku-si (KR).

(74) Agent: **LEE, Won-Hee**; Sung-ji Heights II, 8th Floor, 642-16, Yoksam-dong, Kangnam-ku, Seoul 135-080 (KR).

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(54) Title: TRANSFECTED CELL LINE WHICH CAN BE USED FOR MASS SCREENING OF THE EXPRESSION OF OSTEOBLAST SPECIFIC TRANSCRIPTION FACTOR RUNX2 AND USE THEREOF

(57) Abstract: The present invention relates to expression vector comprising consensus nucleotide sequence of osteoblast specific factor binding element 2 (OSE2) and reporter gene, transfected cell line with said vector and method for screening osteogenesis-promoting materials using said transfected cell line. The transfected cell line of the present invention contains nucleotide sequences targeted by transcription factor Runx2 which can control the differentiation of osteoblast. It allows different expression pattern of reporter gene and quantitative measurement of Runx2 expression in the cell. So, it can be used usefully for the screening of the osteogenesis-promoting materials and for the studying of cellular signal transduction pathway.

Transfected Cell Line Which Can Be Used for Mass Screening of the Expression of Osteoblast Specific Transcription Factor Runx2 and Use Thereof

FIELD OF THE INVENTION

The present invention relates to expression vector comprising consensus nucleotide sequence of osteoblast specific factor binding element 2 (OSE2) and reporter gene, transfected cell line with said vector and method for screening osteogenesis-promoting materials using said transfected cell line. Particularly, it relates to expression vector comprising OSE2 sequence and reporter gene, wherein OSE2 sequence is common to promoters of proteins whose expression indicate the differentiation of osteoblast, transfected cell line with said vector and method for screening osteogenesis-promoting agents using said transfected cell line. The transfected cell line of the present invention contains nucleotide sequences targeted by transcription factor Runx2 which can control the differentiation of osteoblast. It allows different expression pattern of reporter gene and quantitative measurement of Runx2 expression in the cell. So, it can be used usefully for the screening of the osteogenesis-promoting materials and for the studying of cellular signal transduction pathway.

BACKGROUND

Bony tissue is one of the connective tissues comprised of bone cells and extracellular matrices, but is different from other connective tissues in that the ossified connective substances within the extracellular matrices are inorganic. The inorganic substances consist mainly of calcium phosphate which exists as hydroxyapatite crystals $(Ca_{10}(PO_4)_6(OH)_2)$. Bony tissue is hard enough to support and defend against physical stresses of the body. So, its fracture, density reduction or damages attributed by pathogenic changes may cause the body to suffer from deformity. If bony tissue is damaged or removed by any reasons, it has to be regenerated naturally or needs to be substituted with prosthesis or bony materials from another parts of the body by surgery. In addition, healing of the physically broken (fractured) bone or surgically damaged bone requires various prosthetic tools, including artificial bones. In this case, however, it takes a significantly long period of time for the recovery of bone to its original feature and function, so the patient should be suffered from serious physical and mental stresses. Furthermore, as the healing procedure becomes long, the damaged part is increasingly apt to be under

microbial infection. So, perfect curing effect may not be expected.

There remains an urgent need to develop materials to facilitate the healing process (regeneration) of damaged bony tissues caused by osteoporosis, bone fracture or surgery. However, the main purpose of osteogenesis-promoting materials known to date, such as bisphosphonates, calcitonin, estradiol or vitamin D, is to suppress the resorption of bony materials, making them useless for the regeneration of damaged bones. So, there are many efforts to find out new materials which can promote osteogenesis and there needs to develop method for the mass-screening of many materials with less time.

The expression of Runt domain-possessing Runx2 transcription factor (the same name as Cbfa1/Pebp2aA/AML3/Osf2) is known to be essential to the osteoblast differentiation. The expression of Runx2 is restricted to the tissues undergoing active osteogenesis. Osteogenesis is completely suppressed when the gene is deleted by gene knockout techniques (Komori et al., Cell), and the expression of the gene is promoted by cytokines or hormones which can promote osteogenesis (Lee et al., *J. Cell Biochem.*, **1999**, 73, 114-125). That is, the regulation of osteoblast

differentiation is achieved at the highest level on hierarchy by transcription factors such as Dlx5 or Runx2, of which expression is regulated by extracellular signaling materials such as BMP-2, TGF-β1 and FGF2. They can promote differentiation from stem cell to osteoblast by promoting the expression of osteoblast differentiation markers such as osteoclacin, osteopontin, type I collagen and bone sialoprotein. Consequently, transcription activity of Runx2 after drug treatment can be considered to be proportional to osteogenesis-inducing ability of that drug, because Runx2 is transcription factor which can regulate osteogenesis at the highest level on hierarchy and its expression is elevated by the osteogeneis promoting factors. So, the expression of Runx2 can be important criteria to decide osteogenesis-inducing ability by that drug.

Meanwhile, osteoblast specific factor binding element 2 (hereinafter, referred to as "OSE2") exists in the promoter regions of osteoblast specific marker proteins such as osteocalcin, osteopontin and bone sialoprotein etc., and osteoblast specific transcription factor, Runx2, can bind to the element. So, an agent which can increase the expression of Runx2 can also increase all the osteoblast specific promoter activities. That means, if we can determine

the extent of the increase of promoter activity, it can theoretically be possible to screen thematerial that can increase the expression of Runx2, i.e. the osteogenesis-inducing materials. But, their promoter activities did not show remarkable changes by the treatment of Runx2 expression-promoting factors such as BMP-2 or FGF2 (Lee et al., *J. Cell Biochem.*, **1999**, 73, 114-125; Harada H. et al., *J. Biol. Chem.*, **1999**, 274(11), 6972-6978). The reason is that there are several controlling regions in the promoter of several transcription factors more than 1 kb in length, lowering the specificity for Runx2, and the action of several transcription factor are compensated with each other, making it difficult to show remarkable changes of promoter activity. Thus, in this invention, Runx2 specificity and sensitivity of the reporter construct are greatly increased by using 6 concatamers of Runx2 binding site (6xOSE2). In addition, to reduce the time and expense for the repeated transfection of the same reporter vector, and to overcome possible disadvantages originated from inconsistent transfection efficiency in- or between-experiments, a stable cell harboring 6xOSE2-Luc was established for mass-screening.

So, the present inventors constructed expression vector comprising multimers of OSE2 consensus sequence which exists in the promoter region of proteins such

as osteocalcin, type I collagen and bone sialoprotein which are specific-markers for the osteoblast differentiation, and transfected cell line with said expression vector. And, the present inventors completed the presented invention by showing that the transfected cell line of the present invention can be used effectively for the screening of the osteogenesis-promoting materials.

SUMMARY OF THE INVENTION

The object of the present invention is to provide transfected cell line that can easily and quantitatively measure the transcription activity of Runx2, and method for screening osteogenesis-promoting materials using said transfected cell line.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A is a result of Northern blot analysis showing the induction of Runx2 mRNA expression by treatment of 1 ng/ml concentrations of FGF2 in several cell lines.

MC ; MC3T3-E1, ROS ; ROS 17/2.8

Fig. 1B is a result of Northern blot analysis showing the induction of Runx2 mRNA by treatment of several concentrations of FGF2 in MC3T3-E1 cell line.

Fig. 1C is a result of Northern blot analysis showing the induction of Runx2 mRNA in MC3T3-E1 cell line which was treated or was not treated with FGF2 in the presence or absence of a protein synthesis inhibitor, cycloheximide.

CHX ; cycloheximide

Fig. 1D is a result of Northern blot analysis showing the induction of Runx2 mRNA by treatment of FGF2 or FGF4 in MC3T3-E1 cell line.

Fig. 1E is a result of Western blot analysis showing the expression of Runx2 protein by FGF2 treatment.

Fig. 2A is a photograph showing the effect of implanted FGF2-soaked beads on sagittal suture closure in developing mouse calvaria of E15.5 (embryonic day 15.5)in organ culture for 48 hours.

Fig. 2B is a photograph showing the expression of Runx2 by in situ hybridization at the sagittal suture closure of E15.5 mouse transplanted with FGF2- or BSA-soaked beads.

Fig. 3 is a graph showing 6xOSE2 promoter activity in the transiently transfected several cell lines.

■ : control, □ : FGF2

Fig. 4 is a graph showing the influence of several cytokines on 6XOSE2 promoter activity in the transiently transfected C2C12 cell line.

■ : control, □ : FGF2, ▨ : BMP2, ▧ : TGFβ

Fig. 5 is a graph showing the influence of the FGF2 concentrations on 6XOSE2 promoter activity in the transiently transfected C2C12 cell line.

Fig. 6 is a graph showing the influence of the constitutively active FGF2 receptor (FR2C342Y or FR2Y340H) on 6XOSE2 promoter activity in the transiently transfected several cell lines.

Fig. 7A is a graph showing 6XOSE2 promoter activity on the cell line with or without treatment of FGF2, when vector having one of two isotypes of Runx2 (Pebp2αA or Osf2) and 6XOSE2-luc vector of the present invention were co-transfected to the cell line which does not express Runx2.

Fig. 7B is a graph showing 6XOSE2 promoter activity on the cell line, when vector having one of two isotypes of Runx2 (Pebp2αA or Osf2), 6XOSE2-luc vector of the present invention and plasmids having constantly active FGF receptor (FR2C342Y or FR2Y340H) were co-transfected to the cell line which does not express Runx2 (Runx2 -/-).

Fig. 8 is a graph showing 6XOSE2 promoter activity on stably transfected cell clones.

■ : control, □ : FGF2

Fig. 9 is a graph showing 6XOSE2 promoter activity with respect to the numbers of #3 clones.

■ : control, □ : FGF2

Fig. 10 is a graph showing 6XOSE2 promoter

activity with respect to the numbers of #5 clones.

A : 6XOSE2 promoter activity with respect to the number of cells.

B : 6XOSE2 promoter activity was normalized by total cellular proteins (/mg)

■ : control, □ : FGF2

Fig. 11 is a graph showing 6XOSE2 promoter activity with respect to the numbers of #29 clones.

A : 6XOSE2 promoter activity with respect to the number of cells.

B : 6XOSE2 promoter activity per normalized total cellular proteins (1 mg/ml)

■ : control, □ : FGF2

Fig. 12 is a graph showing the influence of FGF2 concentrations on 6XOSE2 promoter activity in #3, #5 and #29 clones.

Fig. 13A is a result of Northern blot analysis showing the expression of Runx2 mRNA induced by FGF2 or FGF4 treatment in the #3 clones stably transfected with p6XOSE2-Luc vector of the present invention.

Fig. 13B is a graph showing relative luciferase activity induced by FGF2 or FGF4 treatment in the #3 clones stably transfected with p6XOSE2-Luc vector.

Fig. 14A is a result of Northern blot analysis showing Runx2 expression and a graph showing luciferase activity in the cell line stably transfected with p6XOSE2-Luc vector which was treated with or without

FGF2 or the inhibitor of Erk1/2 MAP kinase, PD98059(PD).

Fig. 14B is a result of Northern blot analysis showing Runx2 expression and a graph showing luciferase activity in the cell line stably transfected with p6XOSE2-Luc vector which was treated with or without FGF2 or the inhibitor of p38 MAP kinase, SB203580(SB).

Fig. 14C is a result of Northern blot analysis showing Runx2 expression and a graph showing luciferase activity in the cell line which was stably transfected with p6XOSE2-Luc vector and was introduced or was not introduced with expression vector of JNK inhibitor, a dominant negative form of MEKK1 (DN-MEKK1) and was treated with or without FGF2

Fig. 15A is a result of Northern blot analysis showing Runx2 expression in the cell line stably transfected with p6XOSE2-Luc vector with or without treatment of 1 μM PKC inhibitor Calphostin C (Cal C) and 10 ng/ml FGF2.

Fig. 15B is a graph showing luciferase activity in the cell line stably transfected with p6XOSE2-Luc vector with or without treatment of 1 μM PKC inhibitor Calphostin C (Cal C) and 10 ng/ml FGF2.

Fig. 15C is a graph showing luciferase activity in the Runx2(-/-) cell line which does not express Runx2 and was transfected or was not transfected with Runx2-Osf2 expression vector with or without treatment of 1 μM PKC inhibitor Calphostin C (Cal C) and 10 ng/ml FGF2.

Fig. 16 is a diagram showing signal transduction pathway concerning the expression of Runx2 and the activity of Runx2 protein induced by FGF treatment.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

A terminology and technology referred in the present detailed description are used as general meaning of the technical field, which includes the present invention. In addition, references mentioned in the present detailed description are all included in the present detailed description for describing the present invention.

The present invention provides p6XOSE2-Luc expression vector comprising consensus nucleotide sequence of osteoblast specific factor binding element and reporter gene.

The present invention further provides a transfected cell line with said expression vector.

In addition, the present invention provides method for screening osteogenesis-promoting materials using said transfected cell line.

The present invention will be further elucidated hereinafter.

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The present invention provides p6XOSE2-Luc expression vector comprising consensus nucleotide sequence of osteoblast specific factor binding element and reporter gene.

The differentiation of osteoblast should be preceded in order to promote osteogenesis. The expression of various proteins such as osteocalcin, osteopontin, type I collagen and bone sialoprotein represents the differentiation of osteogenesis. There is OSE2 nucleotide sequence which is osteoblast specific element in the promoter region of these genes, and the osteoblast-specific factor, Runx2, is a transcription factor that increases the expression of these genes by binding to the OSE2 sequence.

The present inventors constructed multimer which was made by multiply connecting the consensus sequence of osteoblast specific factor binding region existing in osteocalcin, and a vector comprising said multimer nucleotide sequence and luciferase as a reporter gene. The multimer nucleotide sequence of the present invention is represented preferably by SEQ. ID. NO:1 which comprises consensus nucleotide sequence of osteoblast specific factor binding element, PuACCPuCA, and more preferably is oligomer which comprises said sequence multiply, and most preferably is 6XOSE2 sequence represented by SEQ. ID. NO:2 which contains 6-repeating series of said sequence. The reporter gene

of the present invention can be selected from a group composed of luciferase, β-galactosidase, GFP (green fluorescent protein) and CAT (chloramphenicol acetyltransferase), of which luciferase is preferred.

As above, the present inventors constructed expression vector comprising multimer nucleotide sequence represented by SEQ. ID. No: 2 and luciferase gene as a reporter gene, and named it as "6xOSE2-Luc" vector.

The present invention further provides a transfected cell line with said expression vector.

First, the present inventors investigated the effect of known osteogenesis-promoting materials on the Runx2 expression from several cell lines as a preliminary experiment to confirm whether the transfected cell line with expression vector of the present invention, p6XOSE2-Luc, well reflects the expression of the Runx2 in cells. MC3T3-E1, ROS 17/2.8 and C2C12 is preferable as a host cell, C2C12 being the most preferred cell line.

As a result of measuring the expression of Runx2 mRNA induced by treatment of FGF2 which is known osteogenesis-promoting material, to MC3T3-E1, ROS12/2.8 and C2C12 cell lines, it was found that the Runx2 expression in all of the cell lines treated with FGF2 had highly increased (see Fig. 1A), and Runx2 protein

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was also expressed by translation of Runx2 mRNA (see Fig. 1E). The basal Runx2 mRNA level was high in the osteoblastic cell lines, ROS 17/2.8 and MC3T3-E1 cell, compared with the C2C12 cell line. Especially, Runx2 was highly expressed in the osteosarcoma-originated ROS 17/2.8 cell line. On the other hand, myoblast-originated C2C12 cell line showed lower basal mRNA level, but the extent of the increase of Runx2 mRNA induced by FGF2 treatment was relatively high.

The measurement of the Runx2 expression induced by different concentrations of FGF2 treatment shows that the Runx2 expression was increased proportionally according to FGF2 concentration. However, after adding a certain concentration of FGF2 (10 ng/ml), the Runx2 expression did not show any great differences (See Fig. 1B). Confirming whether the cell lines treated with FGF4 shows the same pattern of increase of Runx2 expression as in the case of FGF2 treatment, it was found that FGF4 could induced the expression of Runx2 mRNA, as in the case of FGF2 (see Fig. 1D).

In result, it was found that the expression of Runx2 mRNA is increasing proportionally with respect to the increased FGF2 concentrations, and FGF2 could induce the expression of Runx2 protein in cells.

To investigate whether Runx2 could be expressed by FGF treatment in vivo, the present inventors performed

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in situ hybridization ex vivo using FGF2-soaked heparin acryl beads. As a result, when FGF2 was treated, tissue volumes were increased and sagittal suture closure was accelerated. It also was observed that Runx2 was specifically expressed around FGF2-treated beads (See Fig. 2).

Runx2 is known to have 2 isoforms, Pebp2αA1 and Osf2(Til-1) (Park et al., *J. Bone Mineral Res.,* **2001**, 16, 885-892). In order to certify which isoforms are induced by the FGF2 treatment, the present inventors performed in situ hybridization using probe specific to each isoforms. As a result, it turned out that both of the isoforms were expressed around the FGF2-soaked beads (see Fig. 2). It means that the FGF2 signal transduction can induce the expression of both Pebp2αA and Osf2.

In order to certify whether the transfected cell line with the p6XOSE2-Luc vector of the present invention reflects well the expression of the Runx2 gene in cells, transiently transfected cell line with p6XOSE2-Luc vector was produced. C2C12, MC3T3-E1 and ROS 17/2.8 cell lines were used as a host cell.

As a result of measuring the Runx2 expression induced by FGF2 treatment to the above transiently transfected various cell lines, it was found that transiently transfected C2C12 cell line showed the highest differences in luciferase activity (see Fig. 3).

In the ROS 17/2.8 cells in which basal Runx2 expression level was found to be the highest by Northern blot analysis (see Fig. 1A), the increase of luciferase activity by FGF2 treatment was not significant since the luciferase activity was already high before FGF2 treatment.

To investigate the effect of cytokines other than FGF2 on the 6XOSE2 promoter activity in transiently transfected several cell lines of the present invention with p6XOSE2-Luc vector, luciferase activity was measured after TGF-β1 and BMP-2 treatment in transfected C2C12 cell line (Lee et al., *J Cell Biochem.*, **1999**, 73, 114-125; Lee et al., *Mol. Cell. Biol.*, **2000**, 20(3), 8783-8792), which showed most differences in luciferase activity. As a result, it was found that luciferase activity was increased in the cells treated with FGF2, however, the cells treated with TGF-β and BMP-2 showed less luciferase activity compared with that of control which had not been treated with FGF2 (see Fig. 4). This indicates that signal transduction pathway in which FGF2 increases Runx2 expression is different from that in which TGF-β or BMP types of cytokines increases Runx2 expression via Smad protein. It also can be distinguishable between the signal transduction pathway which increases Runx2 expression using the transfected cell line of the

present invention and that of which TGF-β or BMP types of cytokines increases Runx2 expression.

In order to find out whether luciferase was expressed by Runx2 in the expression vector of the present invention, the present inventors transfected 6XOSE2 vector of the present invention simultaneously with FR2Y340H or FR2C342Y plasmids to cell line which expresses or does not express Runx2, wherein FR2Y340H or FR2C342Y can constantly expresses FGFR2 mutant protein which always activates FGF2 signal transduction pathway. Then, luciferase activity was measured.

As a result, luciferase activity was increased 2-3 fold in cell lines constantly expresses Runx2 genes without treatment of FGF2. In case of Runx2(-/-) cell lines, FGF2 signal transduction pathway was activated but luciferase activity was not increased (See Fig. 8). In results, it was found that FGF signal transduction can increase luciferase activity via Ruxn2.

Furthermore, in order to certify the effect of different concentrations of FGF2 on Runx2 expression at the transfected cell line of the present invention, the present inventors measured luciferase activity after treatment of different concentrations of FGF2 to the above-transfected C2C12 cell line. As a result, it was found that luciferase activity was increased

proportionally with the added FGF2 concentration (see Fig. 5). In results, it was found that the increase of Runx2 expression which was shown in Northern blot analysis (see Fig. 1B) depends on the FGF2 concentration, and thus 6XOSE2 activity reflects Runx2 expression very well.

From the above results, it is found that the luciferase activity in transiently transfected cell line with p6xOSE-Luc vector of the present invention thoroughly reflects the Runx2 expression and the changes were most prominent in transfected C2C12 cell line. This means that stably transfected C2C12 cell line with p6xOSE2-Luc vector of the present invention can be the most sensitive tool that estimates the Runx2 expression. Therefore the present inventors constructed transfected cell line stably transfected with p6XOSE2-Luc vector of the present invention.

To produce transfected cell line stably with p6XOSE2-Luc vector of the present invention which can be used for mass-screening of osteogenesis-promoting materials that can regulate Runx2 expression and can promote the differentiation of osteoblast, the present inventors co-transfected p6XOSE2-Luc reporter vector with pcDNA3.0 vector (Invitrogen, USA) containing neomycin resistant gene to C2C12 cell line.

As a preferred embodiment to select transfected cell line of the present invention which has been co-transfected with p6xOSE2-Luc vector and a vector containing neomycin resistant gene as a selectable marker, transfected cell line was incubated in the medium containing G418. As a result, the present inventors separated transfected clone stably transfected with p6XOSE2-Luc vector and deposited it at Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology on January 10, 2001 (Accession No: KCTC 0929BP).

As a result of studying the effect of FGF2 on luciferase activity in transfected cell lines having different concentrations of cells, it was found that the more the density of cells was increased, the more the extent of the increase of luciferase activity by FGF2 treatment was decreased (see Fig. 9, Fig. 10A and Fig. 11A). In the case where luciferase activity per total cellular proteins was used to compensate errors caused by the increase of cell numbers, the same patterns were observed (see Fig. 10B and Fig. 11B).

As a result of studying the effect of different concentrations of FGF2 on luciferase activity in

transfected cell lines, it was found that there was no changes of luciferase activity by 1 ng/ml concentrations of FGF2 treatment in all the three types of clones, but strong luciferase activity was found when more than 10 ng/ml concentrations of FGF2 were treated (see Fig. 12), resulting differences compared with the case of Northern blot analysis (see Fig. 1B). However, considering the prior report which evaluates that low concentrations of FGF2 treatment can stimulate cell growth and high concentrations of FGF2 treatment can induce the differentiation of osteoblast cell (Iseki S. et al., *Development*, **1997**, 124, 3375-3384), it can be assumed that such changes could be happen between 1 ng/ml and 10 ng/ml concentrations of FGF concentrations.

In addition, the present invention provides method for screening osteogenesis-promoting materials using said transfected cell line.

The present invention provides a method for mass-screening of materials which can accelerate the differentiation of osteoblast and can increase osteogenesis by measuring Runx2 expression induced by various chemical compounds and natural products using above transfected cell line.

To investigate whether promoter activity of

p6XOSE2 is connected directly with the expression of Runx2 in transfected cell line of the present invention, the present inventors measured luciferase activity and mRNA expression simultaneously in the transfected cell line. As a result, Runx2 mRNA expression and luciferase activity was increased simultaneously in the transfected cell line of the present invention by FGF2 or FGF4 treatment (see, Fig. 13).

In results, it could be assumed that materials which increase luciferase activity when treated into the transfected cell line of the present invention were to increase the expression of Runx2 and such a materials could increase the expression of osteogenesis promoting factors, resulting promotion of the osteoblast differentiation. Therefore, the transfected cell line stably transfected with p6XOSE2-Luc and pcDNA3.0 vector of the present invention could be used usefully for the screening of osteogenesis-promoting materials.

It has reported that FGF/FGFR signal can activate MAP kinase (mitogen-activated protein kinase) and MAP kinase is composed of Erk1/1 MAPKs, p38MAPKs and p54/p46 c-Jun NH2-terminal kinase (JNKs) (Klint and Claesson-Welsh, *Front Biosci.*, **1999**, 4, 165-177; Robinson and Cobb, *Curr. Opin. Cell Bio.*, **1997**, 9, 180-186; Shaeffer and Weber, *Mol. Cell. Biol.*, 19, 2435-

2444). So, the present inventors investigated that which MAPK signal transduction pathway is involved in the induction of Runx2 mRNA expression and Runx2 activation by FGF2 signal transduction pathway. To do this, the transfected cell line of the present invention was treated with FGF2 and each of MAPKs was blocked using signal transduction specific inhibitors. PD98059 was used as a Erk1/2 specific inhibitor, and p38 MAPK signal transduction pathway was blocked by SB203580 treatment. Because the inhibitor for the JNK signal transduction was not obtainable, the present inventors used DN-MEKK-1 (dominant negative MEKK-1) as a inhibitor for the JNK signal transduction pathway (Brown et al., *J. Biol. Chem.*, **1999**, 274, 8797-8805).

As a result, PD98059 could completely inhibited 6XOSE2-Luc reporter activity induced by FGF2 treatment in transfected cell line of the present invention, but could not influence on the Runx2 expression stimulated by FGF2 treatment (see Fig. 14A). p38 MAPK signal transduction which could be inhibited by SB203580 could not influence on the Runx2 expression which was stimulated by FGF2, but it could decrease reporter activities to about 60% of the control, just like the case of Erk1/2 signal transduction (see Fig. 14B). But, the transfection of DN-MEEK-1 in JNK signal transduction neither influenced on the increase of

Runx2 expression by FGF2 treatment nor on 6XOSE2-Luc reporter activity (see Fig. 14C).

In results, it was found that among MAP kinase signal transduction pathways, Erk1/2 or p38 MAPK could increase the transcription activity of Runx2 protein induced by FGF2 treatment, but it could not control the expression of Runx2 mRNA.

The present inventors assumed that the expression of Runx2 mRNA induced by FGF2 treatment is mediated by signal transduction pathway other than MAP kinase signal transduction pathway. Because PKC is activated via FGF/FGFR signal transduction pathway (Klint and Claesson-Welsh, *Front Biosci.*, **1999**, 4, 165-177), the present inventors investigated whether PKC signal transduction is involved in the transcription of Runx2 mRNA stumulated by FGF2 treatment. To do this, the present inventors studied the influence of PKC activity inhibitor, calphostin C, on the expression of Runx2 mRNA.

As a result, it was found that the increase of the expression of Runx2 mRNA induced by FGF2 treatment was not due to the down modulation of PKC activity by calphostin C treatment (see Fig. 15C), and stimulation of 6XOSE2-Luc reporter vector mediated by FGF2 was almost completely disappeared (see Fig. 15B).

In addition, the present inventors investigated whether the increase of luciferase activity induced by FGF2 treatment was only due to the increase of Runx2 mRNA expression or transcription activity of Runx2 protein mediated by PKC was additionally involved.

As a result, it was found that the expression of Runx2 mRNA induced by FGF2 treatment in transfected cell line with 6XOSE2-Luc vector of the present invention was mediated mainly via PKC signal transduction pathway and transcription activity of Runx2 protein was mediated by Erk1/2 or p38 MAPK among MAP kinase signal transduction pathway (see Fig. 16).

Therefore, the transfected cell line with 6xOSE2-Luc vector of the present invention can be used usefully for the investigation of signal transduction pathways by which Runx2 expression could be increased or Runx2 protein could be activated.

EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit

and scope of the present invention.

Referential example 1: Measurement of Runx2 expression induced by FGF treatment

<1-1> Measurement of Runx2 expression induced by FGF2 treatment in cell line

To investigate whether the expression of Runx2 which is crucial to osteoblast differentiation is increasing in cells by the treatment of several transcription factors, the present inventors measured the expression of Runx2 mRNA in the cell lines after treatment of FGF2.

After splitting MC3T3-E1, ROS17/2.8 (Lee M-H. et al., *J. Cell Biochem.*, **1999**, 73, 114-125) and C2C12 cell lines (ATCC, U.S.A.) to the concentrations of 1.1 $\times 10^6$ cells per 100 mm^2, they were incubated in the alpha-MEM medium containing 10% FBS, 10 mM beta-glycerophosphate and 50 μg/ml ascorbic acid, DMEM medium containing 10% FBS and DMEM medium containing 15% FBS, respectively. After they were grown confluently, they were incubated for 24 hours with serum-free medium containing 1 ng/ml concentrations of FGF2, then, the medium was collected.

After that, the present inventors separated total RNA using the collected medium by the method of

Chomczynski and Sacchi (Chomczynski P. and Sacchi N., *Anal. Biochem.*, **1987**, 162, 156-159). In detail, after washing with PBS three times, 1 ml of denaturation solution containing 4 M guanidium thiocyanate was added to 100 mm culture plate. After transferring to a new tube, 0.1 ml of 2 M sodium acetate, 1 ml of phenol and 0.2 ml of chloroform-isoamylalcohol were added to denatured cell solutions, then they were vigorously vortexed. After centrifugation, supernatant was transferred to a new tube and 1 ml of isopropanol was added. After letting them at -20℃ for more than 1 hour, they were centrifuged at 4℃ for 30 minutes with the gravitation of 10,000 g. After centrifugation, isopropanol was removed. After washing with 75% ethanol two times, centrifugation was performed again. After getting rid of ethanol perfectly, RNA was dissolved in DEPC-treated water and RNA was quantified at 260 nm wavelength using spectrophotometer. After electrophoresis of 10 μg of RNA on the gel containing 1% agarose, 55% formaldehyde, they were transferred to zeta-probe membrane (Bio Rad, USA) by capillary phenomenon. Northern blot of transferred membrane was performed using 1×10^6 cpm/ml of alpha ^{32}P-labeled Runx2 cDNA as a probe. Northern blot was performed according to the manufacturer's manual using ExpressHyb hybridization solution (Clontech, CA, USA). After hybridization reaction, transferred membrane was washed

three times at 37℃ for 5 minutes with Sol I containing 0.1% SDS and 2X SSC and then washed again three times with Sol II containing 0.1% SDS and 0.1× SSC at 50℃. After washing the transferred membranes again with 2× SSC, they were exposed to X-ray film (Agfa Co.) and autoradiography was performed.

As a result, it was found that Runx2 expression was strongly increased in all the cell lines treated with FGF2 (Fig. 1A). Basal expression levels of Runx2 mRNA in the osteoblast-originated ROS 17/2.8 or MC3T3-E1 cells were relative high compared with C2C12 cell lines. Especially, osteosarcoma-originated C2C12 cell line had lowered basal expression level, but increase of Runx2 expression level induced by FGF2 treatment was relatively high.

<1-2> Measurement of Runx2 expression level induced by treatment of different concentrations of FGF2

After splitting MC3T3-E1 cell line to the concentrations of 1.1×10^6 cells per 100 mm^2, it was incubated in the alpha-MEM medium containing the same compositions as was the case of above referential example <1-1>. After they were grown confluently, they were incubated for 21 hours with serum-free medium containing 1 ng/ml concentrations of FGF2, then, they were treated again for 3 hours with 0, 0.1, 1, 10 and

30 ng/ml concentrations of FGF2, respectively. After that, the present inventors separated total RNA of cells and performed Northern blot analysis using the same method as the referential example <1-1>.

As a result, it was found that the expression level of Runx2 mRNA was increased proportionally with respect to the increased FGF2 concentrations, and there was no significant differences of Runx2 expression in case of more than 10 ng/ml concentrations of FGF2 was treated (Fig. 1B).

<1-3> Measurement of the effect of protein synthesis inhibitors on Runx2 expression in cells by FGF2 treatment

After splitting MC3T3-E1 cell line to the concentrations of 1.1×10^6 cells per 100 mm^2, it was incubated in the alpha-MEM medium containing the same compositions as was the case of above referential example <1-1>. After they were grown confluently, they were incubated for 21 hours with serum-free medium containing 1 ng/ml concentrations of FGF2, then, they were treated again for 3 hours with 1 ng/ml concentrations of FGF2 and 10 mg/ml concentrations of protein synthesis inhibitor, cycloheximide. After that, the present inventors separated total RNA of cells and performed Northern blot analysis using the same method

as the referential example <1-1>. Cells which do not treated with cycloheximide were used as a control.

As a result, it was found that there was no increase of Runx2 expression in cycloheximide-treated group by FGF2 treatment (Fig. 1C). After pre-treatment of cycloheximide, increased Runx2 expression level by FGF2 treatment was decreased.

In results, it was found that the expression of Runx2 induced by FGF2 treatment, which is crucial transcription factor for osteogenesis, was induced indirectly but dose-dependently by newly-synthesized proteins via FGF2 signal transduction pathway.

<1-4> Measurement of Runx2 expression induced by FGF4 treatment in cell line

To investigate whether the expression of Runx2 also can be increased in cell line by the treatment of FGF4, the present inventors measured the expression of Runx2 mRNA after treatment of FGF2 and FGF4, respectively.

After splitting MC3T3-E1 cell line to the concentrations of 1.1×10^6 cells per 100 mm^2, it was incubated in the alpha-MEM medium containing 10% FBS, 10 mM beta-glycerophoaphate and 50 μg/ml concentrations of ascorbic acid. After they were grown confluently,

they were incubated for 24 hours with serum-free medium containing 1 ng/ml concentrations of FGF2 and FGF4, respectively, then, the medium was collected. After that, the present inventors separated total RNA of cells and performed Northern blot analysis using the same method as the referential example <1-1>.

As a result of this, it was found that FGF4, like FGF2, also could induce the expression of Runx2 mRNA (Fig. 1D).

<1-5> Measurement of the expression of Runx2 protein induced by FGF2 treatment in cell line

To investigate whether the expression of Runx2 protein can be increased in cell line by the treatment of FGF2, the present inventors performed Western blot analysis using monoclonal antibody specific to C-terminal region of Runx2 protein.

MC3T3-E1 cell line with or without treatment of FGF2 was incubated in the alpha-MEM medium containing the same compositions as was the case of above referential example <1-1>. After washing with PBS two times, the pellets were applied to 400 ml of 10 mM Tris-HCl (10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF). After letting them on ice for 15 minutes, 25 ml of 10% NP-40 was added and the suspension were vigorously vortexed. After that, they were centrifuged

for 30 minutes with the gravitation of 12,000 g. After centrifugation, the pellet was re-suspended with 30 ml of Tris-HCl, pH 7.9, and vortexed vigorously at 4℃ for 20 minutes. Cell debris was removed by centrifugation at 4℃ for 15 minutes and the supernatant was collected and stored at -70℃ to be used for electrophoresis. Proteins were quantified using Bradford protein analyzing kit (Bio-Rad), electrophoresized on SDS-PAGE and transferred electrically to Hybond-P membrane (Amersham Pharmacia Biotech). After transferring, blot was washed with PBS at room temperature for 5 minutes and was kept in the blocking buffer (PBS containing 0.1% Tween-20 and 5% nonfat dry milk). After washing the blot with PBS for 5 minutes 3 times, monoclonal antibody specific to C-terminal region of Runx2 protein was diluted to 1:2,000 ratio in blocking buffer and it was added to the blot. Then, it was incubated with slight shaving at room temperature overnight. After washing the blot with PBS 3 times, it was incubated with mouse anti-rabbit antibody at room temperature for 1 hour. After washing with PBS 2 more times, the band was detected using Westzol (iNtRON, Korea).

As a result, more Runx2 protein expression was observed when FGF2 was treated compared with the case of which FGF2 was not treated (Fig. 1E).

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In results, Runx2 mRNA expression was increased proportionally and the expression of Runx2 protein was induced by FGF treatment with dose-dependent manner.

Referential example 2: Runx2 expression by FGF2 treatment ex vivo

To investigate whether the expression of Runx2 protein can be increased ex vivo by the treatment of FGF2, the present inventors performed in situ hybridization using heparin acryl bead soaked with FGF2.

<2-1> Measurement of the expression of Runx2 protein induced by FGF2 treatment in cell line

The present inventors separated the cranium of mouse of E15.5 stage without skin and let them on the filter having pore size of 0.1 μm which is supported by metal grid. After making FGF2-soaked beads by soaking heparin-coated acryl beads (125-150 μm of diameter) in 25 ng/μl of FGF2 or BSA as a control at 37℃ for 30 minutes, the beads were washed and they were transplanted on osteogenic front region of sagittal suture closure using capillary vessel pipette. The explant was incubated in DMEM medium containing penicilline/streptomycin at 37℃, 5% CO_2 for 48 hours.

As a result, it was found that FGF2-soaked beads were expanded with volume and their sealing was accelerated compared with control group treated with BSA-soaked beads (Fig. 2A).

<2-2> in situ hybridization

To perform in situ hybridization, the present inventors used dioxygenin-UTP-labeled riboprobe of sense and antisense Runx2.

The cultured tissue of above referential example <2-1> was treated with proteinase K, re-fixed with PBS containing 4% PFA and 0.2% glutaraldehyde and hybridized using riboprobe at 55℃ overnight. After washing the tissues with 2X SSC solution containing 50% formamide, color reaction was performed using dioxygenin RNA labeling kit (Boeringermannheim, Germany). After color reaction, the tissue was re-fixed and was stored in 50% glycerol solution before taking photograph.

As a result, Runx2 was specifically expressed around FGF2-soaked beads in case of the explant treated with FGF2-soaked bead.

<2-3> The expression of Runx2 isoforms

Runx2 has been known to exist as two types of isoforms, Pebp2αA1 and Osf2(Til-1) (Park et al., *J.*

Bone Mineral Res., **2001**, 16, 885-892). To investigate which isoform is induced by FGF2 stimulation, the present inventors used 3 probes as follows. Using cDNA probe having consensus sequence region of two types of isoforms (pRunx2), probe specific for Pebp2αA (pPebp2αA) or probe specific for Osf2 (pOsf2), in situ hybridization was performed using the same method as the referential example <2-2> (Park et al., *J. Bone Mineral Res.*, **2001**, 16, 885-892).

As a result, it was found that all of the two types of isoforms were expressed around FGF2-soaked beads (Fig. 2B). So, FGF2 signal transduction induced both of the Pebp2αA and Osf2 expression.

Example 1: The construction of expression vector, p6XOSE2-Luc

<1-1> The preparation of 6XOSE2 oligomer

To construct oligomer containing nucleotide sequences which are targeted by one of the osteoblast specific factor, Runx2, the present inventors investigated consensus nucleotide sequences present on the promoter regions of osteocalcin gene of mouse, rat and human origins, mouse collagenase 3 gene and mouse osteopontin gene.

As a result, the present inventors had confirmed that OSE2 nucleotide sequence represented by SEQ. ID. NO:1 was a consensus sequence common to the promoter regions of said osteoblast specific factors, and constructed 6XOSE2 oligomer represented by SEQ. ID. NO:2 having 6 tandem repeat of OSE sequence. In detail, oligomers comprising nucleotide sequence containing OSE2 sequence and its complementary sequence were constructed by ordering from Bioneer Co.(Korea). The complementary sequences were hybridized with equal quantity and ligated by ligase treatment into reaction solution. After that, electrophoresis was performed. Comparing with molecular size marker, 6XOSE2 DNA band which was positioned at 6th position from the smallest monomer was cut off, and 6XOSE2 oligomer DNA represented by SEQ. ID. NO: 2 of the present invention having 168 bp size was separated and purified using DNA elution kit (Promega co.).

<1-2> Preparation of 6XOSE2-Luc reporter vector

To construct expression vector comprising 6XOSE2 oligomer constructed at the above example <1-1>, pGL3 promoter vector (Promega Co.) containing luciferase as a reporter gene was cut off by treatment of XmaI restriction enzyme, and pre-purified 6XOSE2 oligomer was ligated to the DNA fragment. Vector having proper

orientation and luciferase activity was constructed by sequence analysis of cloned vector.

As a result, the present inventors confirmed that 6XOSE2 oligomer fragment was properly inserted into pGL3 promoter vector and referred it as a "p6XOSE2-Luc" vector.

Example 2: The construction of transformant transiently transfected with p6XOSE2-Luc vector

To investigate that p6XOSE2-Luc vector could reflect properly the expression of Runx2 gene in cell, the present inventors constructed transiently transfected cell line with p6XOSE2-Luc vector.

First, C2C12, MC3T3-E1, C3H10T1/2, Runx2(-/-) and ROS17/2.8 cells were split to 1×10^5 cells/well concentrations to 6-well plate and incubated at 5% CO_2 incubator for 24 hours. After incubating them with 0.5 μg of p6XOSE2-Luc reporter vector, 3 μl of PLUS reagent (Gibco BRL. CA, USA) and 100 μl of serum-free DMEM medium for 15 minutes at room temperature, P6XOSE-Luc:lipofectamin complex was generated by mixing with 3 ml of lipofectamin (Gibco BRL. CA, USA) and serum-free DMEM medium. 800 μl of serum-free DMEM medium and 207 μl of p6XOSE-Luc:lipofectamin was added to each well of

incubated C2C12, MC3T3-E1, C3H10T1/2, Runx2(-/-) and ROS17/2.8 cells and incubated them at 5% CO_2 incubator for 3 hours. By doing such, transiently transfected cell lines with p6XOSE2-Luc vector of the present invention was constructed.

<2-1> Measurement of luciferase activity induced by FGF2 treatment in transfected cell lines

To investigate whether FGF/FGFR signal transduction pathway could stimulate the transcriptional activities mediated by Runx2, the present inventors treated 10.0 ng/ml concentrations of FGF2 to each of transiently transfected cell lines constructed by above method. After incubation at 5% CO_2 incubator for 24 hours, luciferase activity was measured using luciferase analyzing kit (Promega Co.).

As a result, it was found that there was no differences on luciferase activity in Runx2(-/-) cells which could not express Runx2, and most changes on luciferase activity was found in transiently transfected C2C12 cell line (Fig. 3). In case of ROS17/2.8 cells which showed highest basal level of Runx2 expression in Northern blot analysis (see Fig. 1A), there were no significant differences on luciferase activity induced by FGF2 treatment because it already showed higher luciferase activity before

FGF2 treatment.

<2-2> Measurement of the effect of cytokine treatment on luciferase activity in transfected cell lines

To investigate the influence of other cytokines which had known to increase the Runx2 expression on 6XOSE2 promoter activity of transfected cell lines, the present inventors treated TGF-β1 and BMP-2 which were known to increase Runx2 expression to C2C12 transformant which showed the highest differences of luciferase activity at the above referential example <2-1>, and measured luciferase activity(Lee et al., *J. Cell Biochem.*, **1999**, 73, 114-125; Lee et al, *Mol. Cell Biol.*, **2000**, 20(3), 8783-8792).

First, C2C12 transfected cell line was treated with 2 ml of FGF2, BMP-2 and TGF-β1 of which concentrations was 2 ng/ml, 300 ng/ml and 5 ng/ml, respectively. After incubation for 24 hours, luciferase activity was measured.

As a result, it was found that luciferase activity was increased in the transformant treated with FGF2, but luciferase activity was decreased in case of TGF-β1 and BMP-2-treated transformant compared with control group (Fig. 4). In results, it was found that signal transduction pathway by which FGF2 increases Runx2 expression was different from that by which cytokine of

TGF-β or BMP family, for example, Smad etc., increases Runx2 expression.

<2-3> Measurement of luciferase activity induced by the treatment of different concentrations of FGF2

To investigate the effect of the treatment of different concentrations of FGF2 on luciferase activity in above trasnfected cell line, the present inventors treated 0, 0.1, 1, 10, 30 and 50 ng/ml concentrations of FGF2 into 2×10^5 cells/well of above C2C12 transformant. After incubation at 5% CO_2 incubators for 24 hours, luciferase activity was measured using luciferase activity analyzing kit.

As a result, luciferase activity was increased proportionally in accordance with added FGF2 concentrations (Fig. 5). In results, it was found that the increase of Runx2 expression observed in the above referential example <1-2> was dependent on the concentrations of FGF2 treatment.

<2-4> Measurement of the increase of luciferase activity by Runx2

To investigate whether luciferase was expressed by Runx2 expression in transfected cell line of the present invention, the present inventors transfected 6XOSE2 vector of the present invention simultaneously

with FR2Y340H or FR2C342Y plasmid to cells which express or do not express Runx2, wherein FR2Y340H or FR2C342Y can constantly expresses FGFR2 mutant protein which always activates FGF2 signal transduction pathway. Then, luciferase activity was measured.

First, Runx2(-/-), MC3T3-E1, C3H10T1/2 and C2C12 cells were split to 1× 10^5 cells/well concentrations to 6-well plate and incubated at 5% CO_2 incubator for 24 hours. They were mixed with 0.5 μg of p6XOSE2-Luc vector, 0.5 μg of pFR2Y340H, 3 μl of PLUS reagent and 100 μl of serum-free DMEM medium and incubated for 15 minutes at room temperature. After preparing mixture of 3 μl of PLUS reagent and 100 μl of serum-free DMEM medium, it was re-mixed with mixture of either p6xOSE2-Luc and pFR2Y340H vectors or p6XOSE2-Luc and pFR2C342Y vectors, generating p6XOSE-Luc:pFR2Y340H:lipofectamin or p6XOSE-Luc:pFR2C342Y:lipofectamin complexes. 800 μl of serum-free DMEM medium and either of 207 μl of p6XOSE-Luc:pFR2Y340H:lipofectamin complex or p6XOSE-Luc:pFR2C342Y:lipofectamin complex were added to each well of incubated C2C12, MC3T3-E1, C3H10T1/2, Runx2(-/-) and ROS17/2.8 cells and they were incubated at 5% CO_2 incubator for 3 hours. By doing such, transiently transfected cell lines simultaneously with p6XOSE2-Luc vector of the present invention and either of pFR2Y340H or pFR2C342Y vector was constructed. Control group was

transfected with pcDNA3.1 vector instead of pFR2Y340H vector.

As a result, luciferase activity was increased 2-3 fold in cell lines constantly expresses Runx2 genes without treatment of FGF2. In case of Runx2(-/-) cell lines, FGF2 signal transduction pathway was activated but liciferase activity was not increased (Fig. 8). In results, it was found that FGF signal transduction can increase luciferase activity via Ruxn2 and the activity of 6XOSE2 can reflect the expression of Runx2 well.

Example 3: The increase of transcription activity of Runx2 by FGF treatment

The present inventors investigated whether luciferase activity caused by the increase of the transcription activity of Runx2 is by the increase of the expression of Runx2 mRNA or by the increase of transcription activity of Runx2 protein.

To do this, the present inventors transfected plasmid containing two isotypes of Runx2 gene to Runx2(-/-) cell line which does not express Runx2. In detail, p6XOSE2-Luc vector and either of Runx2-pebp2aA expression vector or Runx2-osf2 expression vector were co-transfected to Runx2(-/-) cell line. After

treatment of 10.0 ng/ml concentrations of FGF2, cells were incubated at 5% CO_2 incubator for 24 hours and luciferase activity was measured using luciferase analyzing kit (Promega Co.).

As a result, luciferase activity was increased by the expression of Runx2 isotypes in Runx2(-/-) cell line (Fig. 7). It also was found that more transcription activity was observed in case of Runx2-osf2 transfection than in case of Runx2-pdbp2aA transfection. Transfected cell line simultaneously with p6XOSE2-Luc vector and either of Runx2-pebp2aA or Runx2-osf2 expression vector showed the induction of the increase of luciferase activity by FGF2 treatment (Fig. 7A). In addition, when p6XOSE2-Luc reporter vector, one of the two Runx2 expression vectors and FGFR2 expression vector (FR2Y340H or FR2C342Y) were co-transfected to Runx2(-/-) cell line, the activity of p6XOSE2-luc was increased (Fig. 7B). In results, FGF/FGFR signal transduction can not only stimulate Runx2 mRNA expression, but also increase the transcription activity of Runx2 protein in cell.

Example 4: The construction of transfected cell line stably with p6XOSE2-Luc vector

The present inventors have confirmed that luciferase activity in cells transiently transfected with p6XOSE2-Luc vector can reflect Runx2 expression well and the changes were most prominent in C2C12 cell line. So, the present inventors assumed that C2C12 cell line stably transfected with p6XOSE2-Luc vector can be used as most sensitive standard to measure the expression of Runx2, and constructed transfected cell line stably with above p6XOSE2-Luc vector.

To construct transfected cell line stably with p6XOSE2-Luc vector which can be used mass-screening of osteogenesis-promoting materials, the present inventors constructed co-transfected C2C12 cell line with pcDNA3.0 vector (Invitrogen, USA) containing neomycin resistant gene and p6XOSE2-Luc reporter vector.

First, C2C12 cells were split to 2×10^5 cells/well concentrations to 100 mm cell culture plate and incubated at 5% CO_2 incubator for 24 hours. They were incubated with 10 μg of p6XOSE2-Luc reporter vector, 2 μg of pcDNA3.0 vector, 15 μl of PLUS reagent and 750 μl of serum-free DMEM medium for 15 minutes at room temperature. After preparing mixtures of 20 μl of

lipofectamin and 750 μl of serum-free DMEM medium, p6XOSE2-Luc:lipofectamin complex was made by re-mixing it with the mixture of p6XOSE2-Luc reporter vector and pcDNA3.0 vector. 5 ml of serum-free DMEM medium and 1,535 μl of p6XOSE2-Luc:lipofectamin complex were added to C2C12 cell line and incubated them at 5% CO_2 incubator for 3 hours. Then, 6.5 ml of DMEM medium containing 30% FBS was added to plate and incubated it at 5% CO_2 incubator for 24 hours. After removing culture medium and collecting cells by trypsin treatment, the cells were cultured in the 100 mm plate to the dilution of 1:1000, 1:5000 and 1:10000 ratio with DMEM medium containing 15% of serum, then they were incubated at 5% CO_2 incubator for 24 hours. After removing culture medium, DMEM medium containing 2 mg/ml concentrations of G418 (neomycin) and 15% of FBS was added to plate and they were incubated at 5% CO_2 incubator for 24 hours. After removing culture medium containing G418, new culture medium containing G418 was added to the plate. After washing the plate with PBS solution, the clones were separated using separation ring and were split in order to be one clone per each well of 12 well plate. After adding culture medium which does not contain G418 to each well, they were incubated at 5% CO_2 incubator for 24 hours.

Because normal C2C12 cells will die 100% under 2

mg/ml concentrations of G418 treatment but cells transfected with pcDNA3.0 which has a gene capable of utilize G418 can survive under the same circumstances, the present inventors tried to select transfected cells simultaneously with p6XOSE2-Luc reporter vector and pcDNA3.0 vector by incubating cells with DMEM medium containing 2.5 mg/ml concentrations of G418 and 15% FBS. After removing culture medium which does not contain G418, DMEM medium containing 2.5 mg/ml concentrations of G418 and 15% FBS was added to cells and they were incubated at 5% CO_2 incubator until clones were formed. Each clone separated from 12-well plate was passaged to 6-well plate and each clone separated from above 6-well plate was passaged again to 100 mm plate. After that, the present inventors separated about 60 clones transfected stably with p6XOSE2-Luc vector of the present invention.

<4-1> Measurement of the luciferase activity induced by FGF2 treatment

To measure luciferase activity induced by FGF2 treatment in transfected cells stably with p6XOSE2-Luc and pcDNA3.0 vectors, the present inventors inoculated above transfected clones into 6-well plate to a concentrations of 2×10^5 cell/well and incubated at 5% CO_2 incubator for 24 hours. After addition of 10 ng/ml concentrations of FGF2 to each culture plate, it was

incubated at 5% CO_2 incubator for 24 hours. Then, luciferase activity was measured using luciferase analyzing kit (Table 1 and Fig. 8)

<Table 1> 6XOSE2 promoter activity in stably transfected clones

Clone No.	Control*	S.D(±)	Sample**	S.D(±)	Ratio (FGF2/no FGF2)
# 1	167.3	33.3	301.7	31.5	1.8
# 3	2686.3	49.9	110397.3	3535.0	41.1
# 4	3568.7	98.3	10287.7	1311.3	2.9
# 5	8711.3	466.0	134590.3	8044.8	15.5
# 6	3279.7	388.2	10381.3	1182.3	3.2
# 7	62619.3	3279.2	130726.0	4324.7	2.1
# 9	1505.3	98.4	8556.3	1140.3	5.7
#11	217.3	129.5	1394.3	58.6	6.4
#15	466.0	343.0	640.7	412.2	1.4
#19	612.7	33.1	692.0	57.0	1.1
#20	14857.7	798.2	36746.0	2733.3	2.5
#21	56.7	6.8	85.3	8.1	1.5
#22	358.7	17.0	418.3	42.0	1.2
#23	258.3	22.8	1380.7	140.3	5.3
#24	2581.0	258.6	6282.0	259.1	2.4
#25	15466.3	638.5	76351.0	3746.6	4.9
#27	1240.3	42.7	5153.0	282.2	4.2
#28	10129.0	118.0	22273.7	2497.7	2.2
#29	2104.7	52.8	16370.7	629.0	7.8

* : relative luciferase activity on average when FGF was not treated.

** : relative luciferase activity on average when FGF was treated.

As a result, it was found that about 20 clones were selected having luciferase activity. Among these, three clones (#3, #5 and #29) which had strongly increased luciferase activity by FGF treatment were

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selected and used in experiments hereinafter (Table 1 and Fig. 8). In case of #3 clone, there found about 40-fold increase of luciferase activity by FGF2 treatment and the basal activity of #3 clone was maintained relatively high. In case of #5 clone, it had relatively higher basal activity and showed 15-fold increase on average by FGF2 treatment. On the contrary, in case of #29, it had relatively lower basal activity and showed 7- or 8-fold increase by FGF2 treatment (Table 1).

<4-2> Measurement of the effect of the cell concentrations on luciferase activity

To investigate the effect of FGF2 treatment on luciferase activity in the transfected cell lines having different concentrations of cell numbers, 10.0 ng/ml concentrations of FGF2 was treated to 0.2×10^5, 1.0×10^5 and 10×10^5 cells/well concentration of each #3, #5 and #29 clones. After incubating them at 5% CO_2 incubator for 24 hours, luciferase activity was measured using luciferase analyzing kit. In order to compensate errors caused by increased cell numbers, total cellular proteins were measured using BCA protein assay kit (Pierce Chemical Co., Rockford, USA), making total luciferase activity per total cellular proteins.

As a result, it was found that the increasing rate

```
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of luciferase activity by FGF2 treatment was decreased with respect to increased cellular density (Fig. 9, Fig 10A and Fig 11A). However, in case of #3 clone, there still found more than 10-fold increase of luciferase activity (Fig. 9). In case of #5 and #29 clones, the increasing rate of luciferase activity was sharply decreased to only 2-3 folds, when cell were exceeded the confluent state (Fig 10A and Fig. 11A). In case of compensation with total luciferase activity per total cellualr proteins in order to compensate errors with increased cell numbers, the same tendency was also observed (Fig. 10B and Fig. 11B).

<4-3> Measurement of luciferase activity induced by different concentrations of FGF2 treatment

To investigate the effect of different concentrations of FGF2 treatment on luciferase activity in the transfected cell lines, 0, 0.1, 1, 10 and 30 ng/ml concentrations of FGF2 were treated to 2×10^5 cells/well concentration of each #3, #5 and #29 clones. After incubating them at 5% CO_2 incubator for 24 hours, luciferase activity was measured using luciferase analyzing kit.

As a result, it was found that there was no changes of luciferase activity by 1 ng/ml concentrations of FGF2 treatment in all the three

clones, but strong luciferase activity was found when more than 10 ng/ml concentrations of FGF2 were treated (Fig. 12), resulting differences compared with the case of Northern blot analysis (see Fig. 1B). However, considering the prior report which evaluates that low concentrations of FGF2 treatment can stimulate cell growth and high concentrations of FGF2 treatment can induce the differentiation of osteoblast cell (Iseki S. et al., *Development*, **1997**, 124, 3375-3384), it can be assumed that such changes could be happen between 1 ng/ml and 10 ng/ml concentrations of FGF concentrations.

In results, the present inventors concluded that #3 clone was the best cell lines which can be used for the screening of osteogenesis stimulating factors and deposited that at Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology on January, 10, 2001 (Accession No: KCTC 0929BP).

Example 5: The relationship between luciferase activity and the expression of Runx2

To investigate whether promoter activity of p6XOSE2 is connected directly with the expression of Runx2 in #3 clone of the present invention, the present

inventors measured luciferase activity and mRNA expression simultaneously in #3 clone.

First, 10.0 ng/ml concentrations of FGF2 was treated to 2.0×10^5 cells/well concentrations of #3 clone and the cells were incubated at 5% CO_2 incubator for 24 hours. After that, luciferase activity was measured using luciferase analyzing kit and Northern blot analysis was performed by the method of referential example <1-1>.

As a result, Runx2 mRNA expression and luciferase activity was increased simultaneously in #3 clone of the present invention by FGF2 or FGF4 treatment (Fig. 13).

In results, it could be assumed that materials which could increase luciferase activity when treated into #3 clone of the present invention were to increase the expression of Runx2 and such a materials could increase the expression of osteogenesis promoting factors, resulting acceleration of the osteoblast differentiation. Therefore, #3 clone stably transfected with p6XOSE2-Luc and pcDNA3.0 vector of the present invention could be used usefully for the screening of osteogenesis-promoting materials.

Example 6: Other signal transduction pathway involved in Runx2 mRNA expression and Runx2 transcription activity induced by FGF treatment

It was reported that FGF/FGFR signal could activate MAP kinase (mitogen-activated protein kinase) and MAP kinase was composed of Erkl/1 MAPKs, p38MAPKs and p54/p46 c-Jun NH2-terminal kinase (JNKs) (Klint and Claesson-Welsh, *Front Biosci.*, **1999**, 4, 165-177; Robinson and Cobb, *Curr. Opin. Cell Bio.*, **1997**, 9, 180-186; Shaeffer and Weber, *Mol. Cell. Biol.*, 19, 2435-2444). So, the present inventors investigated that which MAPK signal transduction pathway is involved in the induction of Runx2 mRNA expression and Runx2 activation by FGF2 signal transduction pathway. To do this, #3 clone of the present invention was treated with FGF2, and each of MAPKs was blocked using signal transduction specific inhibitor. PD98059 was used as a Erkl/2 specific inhibitor, and p38 MAPK signal transduction pathway was blocked by SB203580 treatment. Because the inhibitor for the JNK signal transduction was not obtainable, the present inventors used DN-MEKK-1 (dominant negative MEKK-1) as a inhibitor for the JNK signal transduction pathway (Brown et al., *J. Biol. Chem.*, **1999**, 274, 8797-8805). To study proper concentration of inhibitor in C2C12 cells, cytotoxicity

by the inhibitor was tested by MTT analysis (Thykjaer T, Christensen M, Clark AB, Hansen LR, Kunkel TA, Orntoft TF., *Br. J. Cancer.*, **2001**, 85(4), 568-575) and luciferase activity was measured. Maximal concentration which does not effect to cytotoxicity was used as inhibitor concentration of the present invention.

As a result, PD98059 could completely inhibited 6XOSE2-Luc reporter activity induced by FGF2 treatment in transfected cell line of the present invention, but could not influence on the Runx2 expression stimulated by FGF2 treatment (Fig. 14A). p38MAPK signal transduction which could be inhibited by SB203580 could not influence on the Runx2 expression which was stimulated by FGF2, but it could decrease reporter activities to about 60% of the control, just like the case of Erk1/2 signal transduction (Fig. 14B). But, the transfection of DN-MEEK-1 in JNK signal transduction increased basal Runx2 mRNA level and did not influenced on the increase of Runx2 expression by FGF2 treatment. In addition, the transfection of DN-MEEK-1 did not inhibited 6XOSE2-Luc reporter activity, which was consistent with the case of Northern blot analysis (Fig. 14C).

In results, it was found that among MAP kinase

signal transduction pathways, Erk1/2 or p38 MAPK could increase the transcription activity of Runx2 protein induced by FGF2 treatment, but it could not control the expression of Runx2 mRNA.

Example 7: The expression of Runx2 induced by FGF2 treatment via PKC signal transduction

The present inventors assumed that the expression of Runx2 mRNA induced by FGF2 treatment is mediated by signal transduction pathway other than MAP kinase signal transduction pathway. Because PKC is activated via FGF/FGFR signal transduction pathway (Klint and Claesson-Welsh, *Front Biosci.*, **1999**, 4, 165-177), the present inventors investigated whether PKC signal transduction is involved in the transcription of Runx2 mRNA stumulated by FGF2 treatment. To do this, the present inventors studied the influence of PKC activity inhibitor, calphostin C, on the expression of Runx2 mRNA. In detail, after incubating cells with DMEM medium which does not contain FBS for 6 hours, PKC activity inhibitor, calphostin C, was treated for 3 hours, blocking all the residual PKC activity. After that, 0 or 10 ng/ml concentrations of FGF2 was added to the medium and incubated 3 more hours then cells were collected. After that, the expression of Runx2 mRNA

was quantified by Northern blot analysis or by the measurement of luciferase activity.

As a result, it was found that the increase of the expression of Runx2 mRNA induced by FGF2 treatment was not due to the down modulation of PKC activity by calphostin C treatment (Fig. 15C), and stimulation of 6XOSE2-Luc reporter vector mediated by FGF2 was almost completely disappeared (Fig. 15B).

In addition, the present inventors investigated whether the increase of luciferase activity induced by FGF2 treatment was only due to the increase of Runx2 mRNA expression or transcription activity of Runx2 protein mediated by PKC was additionally involved.

As was seen in the result of above example 3, over-expression of Runx2-osf2 in Runx2(-/-) cells resulted in the increase of luciferase activity, and FGF2 treatment in those cells resulted in the even more increase of luciferase activity (Fig. 7). But, such a increased luciferase activity by FGF2 treatment was almost disappeared by blocking PKC signal transduction by pre-treatment of calphostin C (Fig. 15C). However, when co-transfected with Runx2-osf2 expression vector, luciferase activity was somewhat increased by the Runx2 expression.

In result, it was found that the expression of Runx2 mRNA induced by FGF2 treatment in transfected cell line with 6XOSE2-Luc vector of the present invention was mediated mainly via PKC signal transduction pathway and transcription activity of Runx2 protein was mediated by Erk1/2 or p38 MAPK among MAP kinase signal transduction pathway (Fig. 16).

INDUSTRIAL APPLICABILITY

Transfected cell line of the present invention was constructed by transfection with vector comprising consensus nucleotide sequence common to promoter region of the pretein which can stimulate the differentiation of osteoblaset and reporter gene and it has high specificity for soteoblast specific factors. So, it can be used usefully for the screening of osteogenesis stimulating materials.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit

and scope of the invention as set forth in the appended claims.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : RYOO, Hyun-Mo
Dep. of Biochemistry, School of Dentistry, Kyungpook National University,
#101, Dongin-dong, Jung-ku, Taegu 700-422,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
C2C12-6XOSE-Luc (mouse myoblastic cell line)	**KCTC 0929BP**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **January 05 2001.**

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s): Bae K S BAE, Kyung Sook, Director Date: January 10 2001

What is Claimed is

1. Expression vector comprising consensus nucleotide sequence of osteoblast specific factor binding element 2, OSE2, and reporter gene, wherein OSE2 is existing at the promoter region of genes stimulating differentiation of osteoblast.

2. Expression vector according to claim 1, wherein consensus nucleotide sequence of osteoblast specific factor binding element 2 is oligomer of nucleotide sequence represented by SEQ. ID. NO:1.

3. Expression vector according to claim 2, wherein oligomer is 6XOSE2 represented by SEQ. ID. NO:2.

4. Expression vector according to claim 1, wherein reporter gene is selected from a group consisting luciferase, β-galactosidase, GFP (green fluorescent protein) and CAT (chloramphenicol acetyltransferase).

5. Expression vector according to claim 1, wherein the expression vector is p6XOSE2-Luc vector which is comprising 6XOSE2 oligomer and luciferase gene.

6. Transfected cell line constructed by transfection of

expression vector of claim 1 to host cell.

7. Transfected cell line according to claim 6, wherein host cell is selected from a group consisting MC3T3-E1, ROS 17/2.8 and C2C12 cell lines.

8. Transfected cell line according to claim 6, wherein it is constructed by transfection of p6XOSE2-Luc expression vector to C2C12 cell line (Accession No.: KCTC 0929BP).

9. Method for measuring the quantity of Runx2 expression by measurement of luciferase activity in transfected cell line of claim 6.

10. Method for screening of osteogenesis stimulating materials by measurement of Runx2 expression after treatment of 임의의 chemical compounds and natural products.

11. Method for screening of signal transduction pathway which can increase the expression of Runx2 mRNA ro can activate Runx2 protein via FGF signal transduction pathway using transfected cell line of claim 6.

FIGURES

FIG. 1

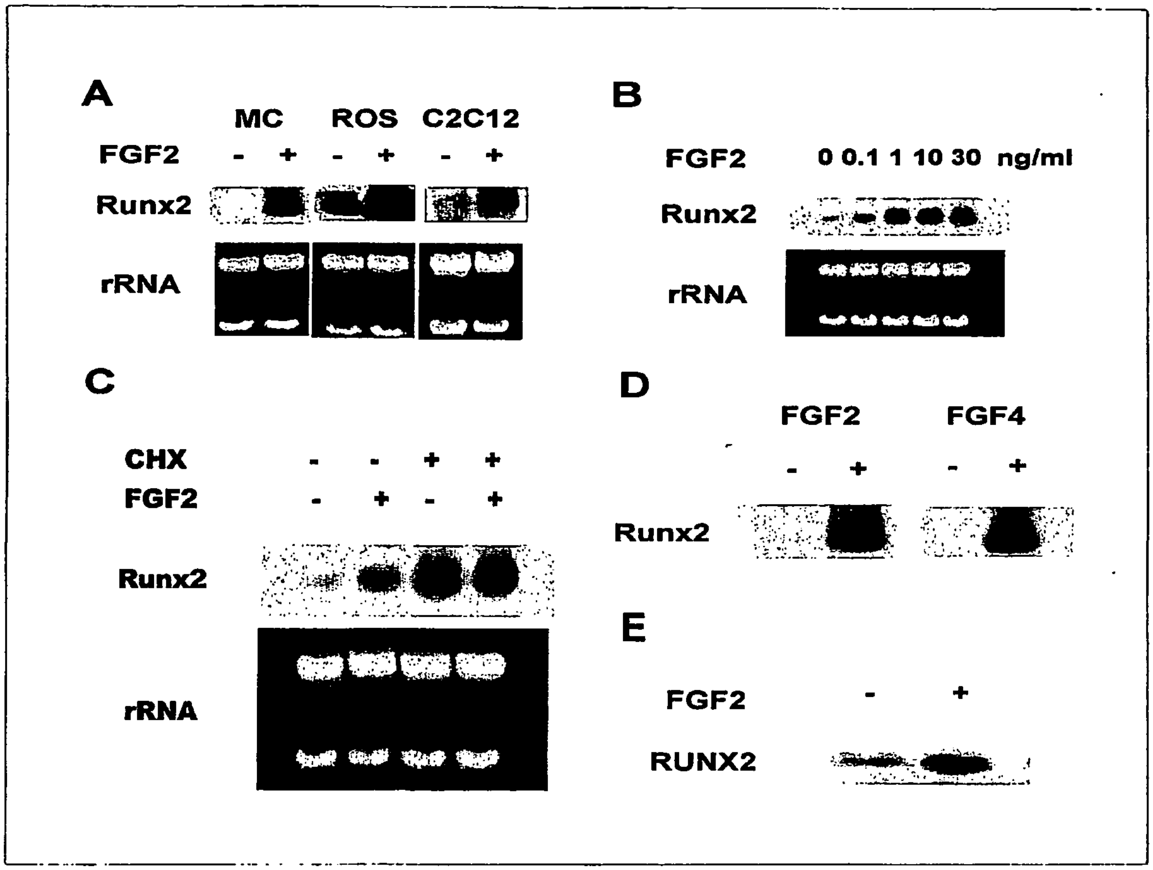

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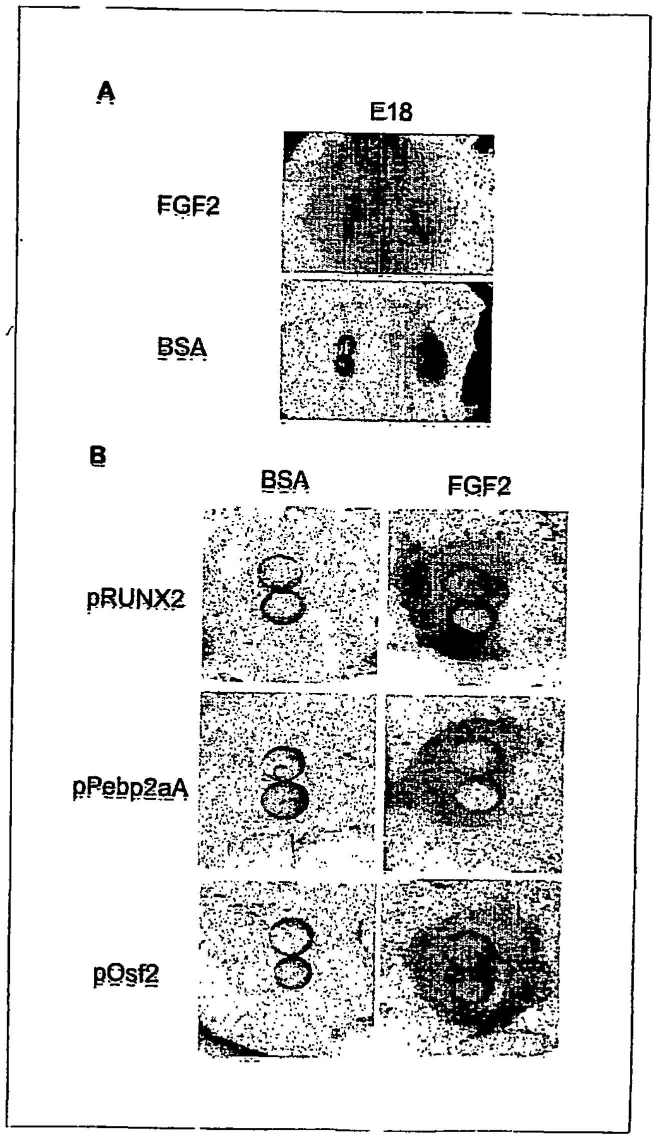
FIG. 2



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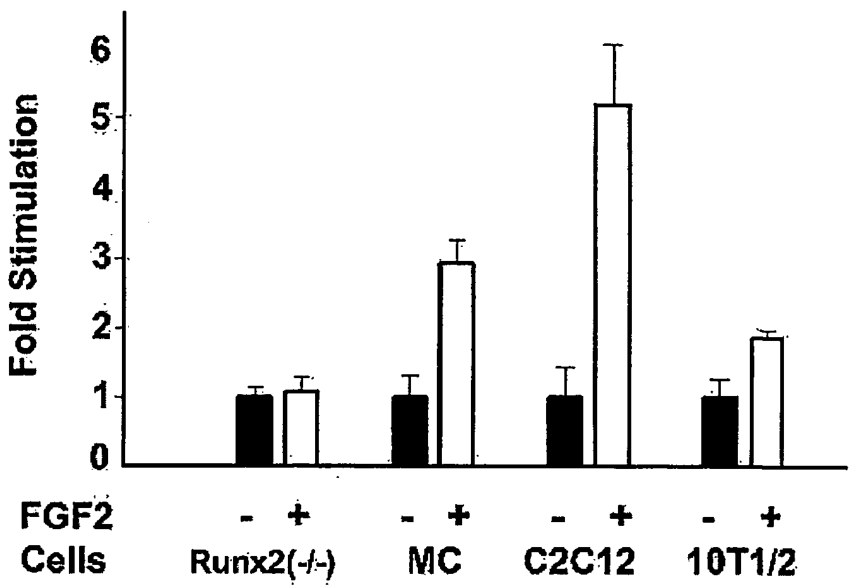

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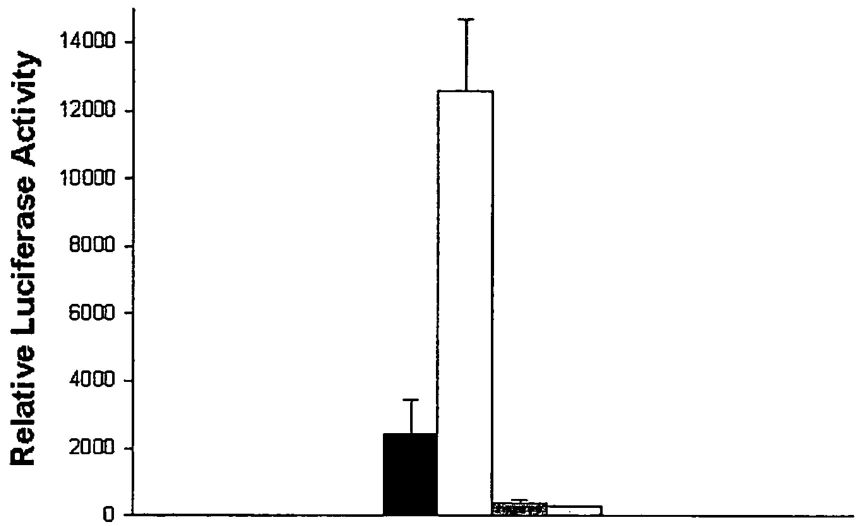
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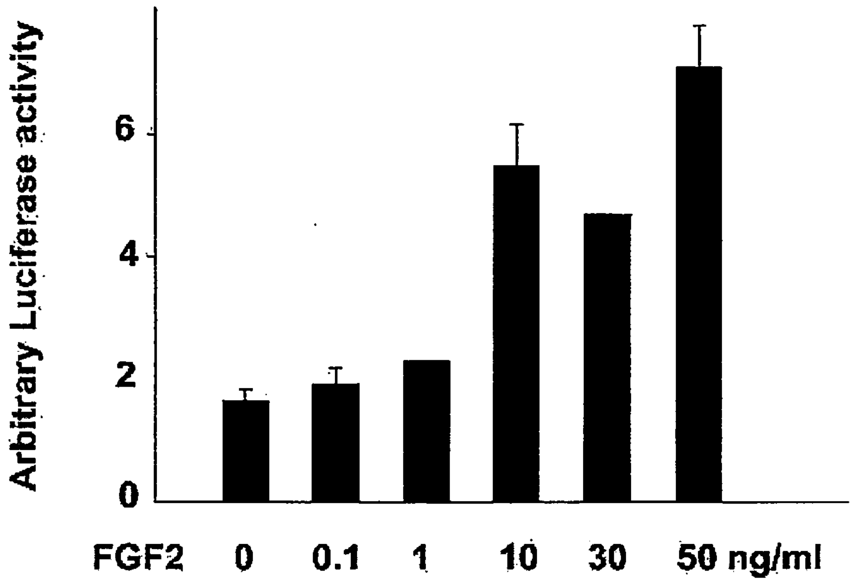
FIG. 5



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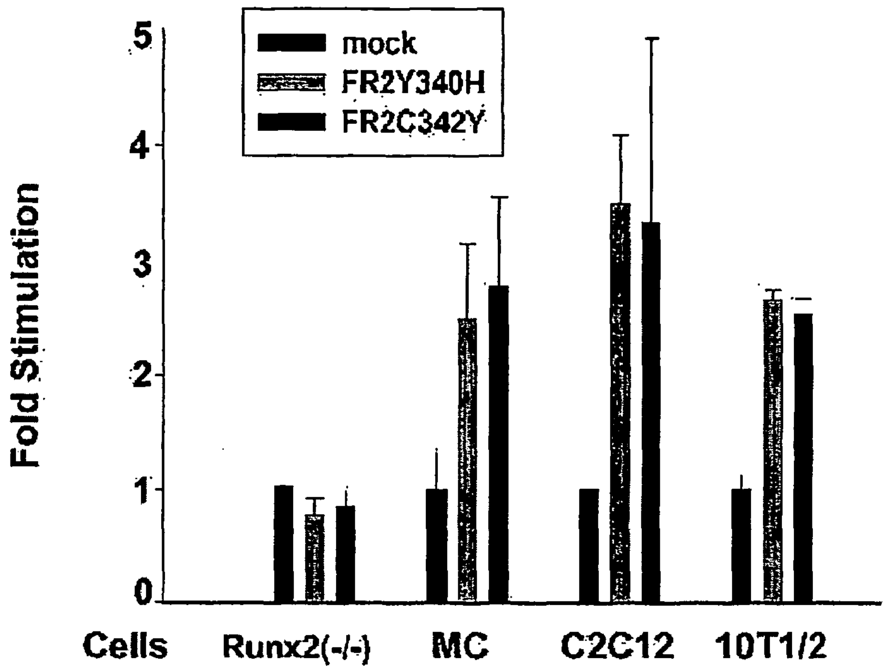
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FR2C342Y
Fold Stimulation
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Cells
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MC
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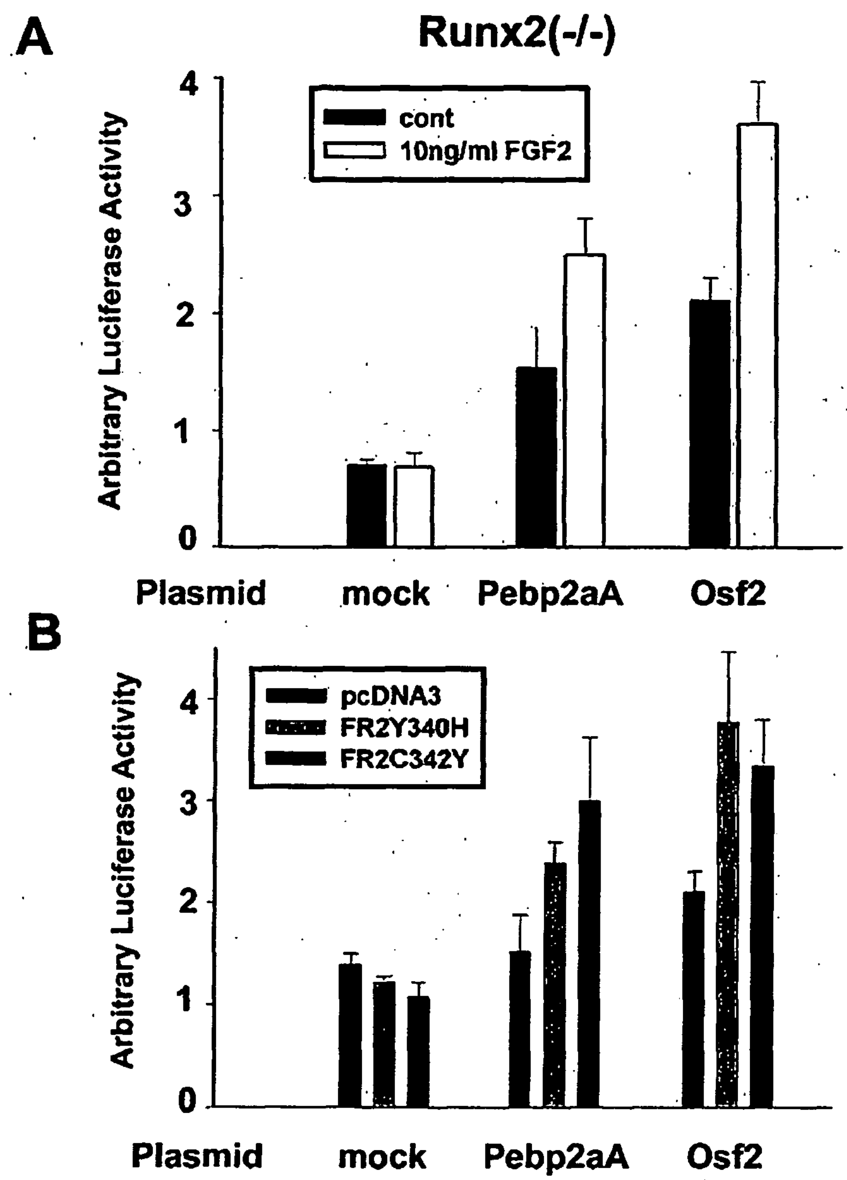
10T1/2

FIG. 7


A
Runx2(-/-)
Arbitrary Luciferase Activity
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1
2
3
4
cont
10ng/ml FGF2
Plasmid
mock
Pebp2aA
Osf2
B
Arbitrary Luciferase Activity
pcDNA3
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FR2C342Y
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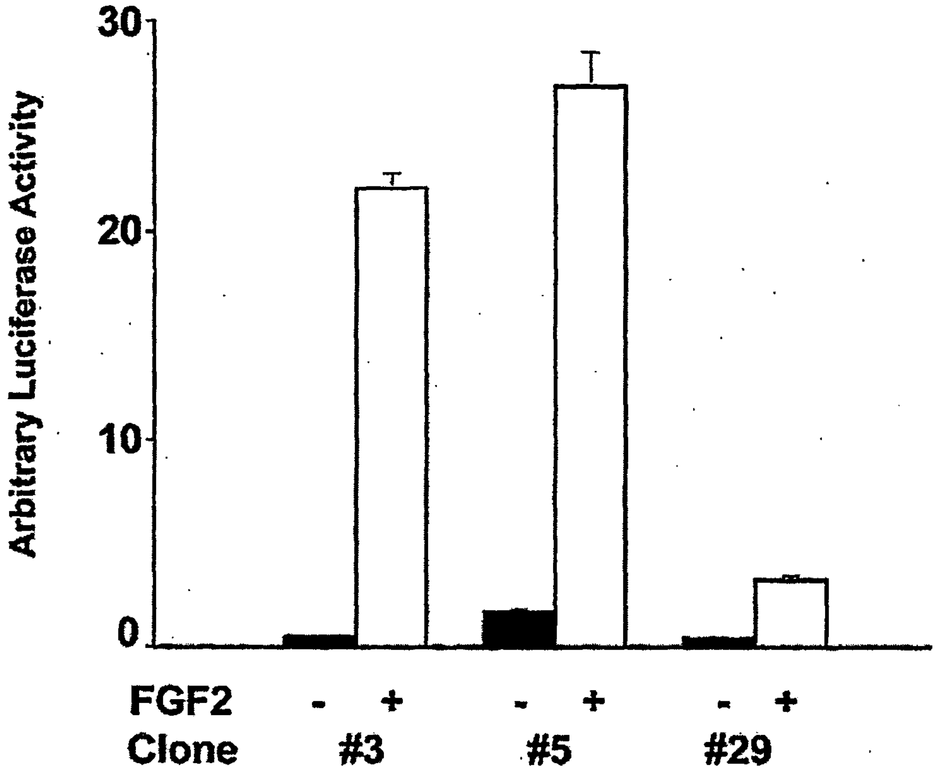
1
2
3
4
Plasmid
mock
Pebp2aA
Osf2

FIG. 8



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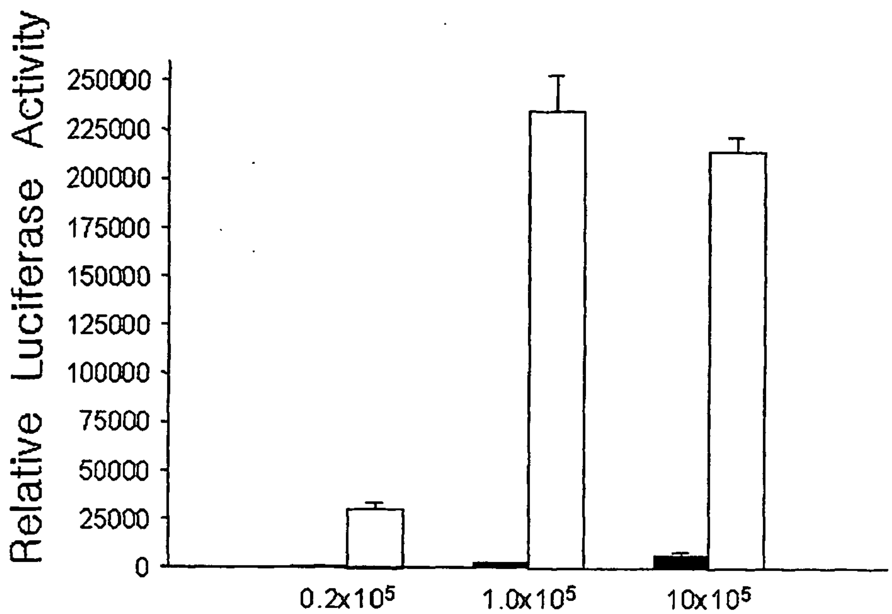
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200000
175000
150000
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75000
50000
25000
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1.0x10⁵
10x10⁵

FIG. 10

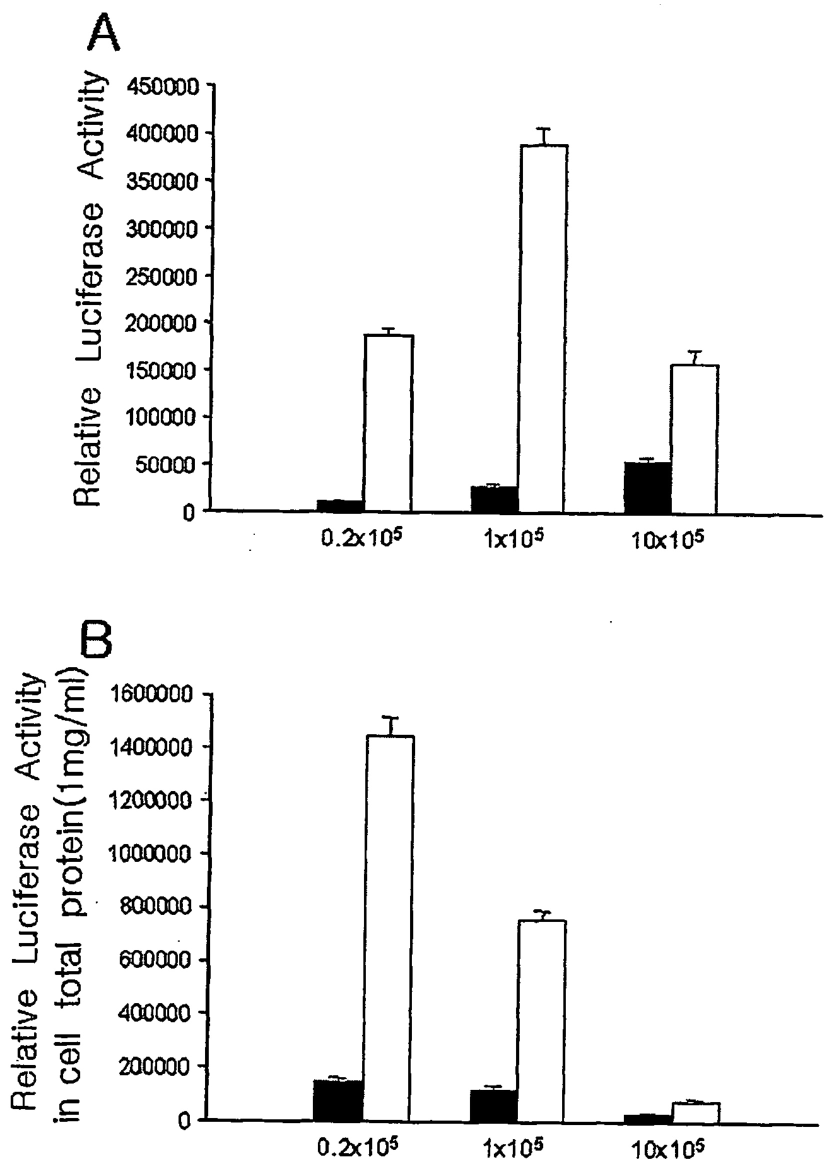
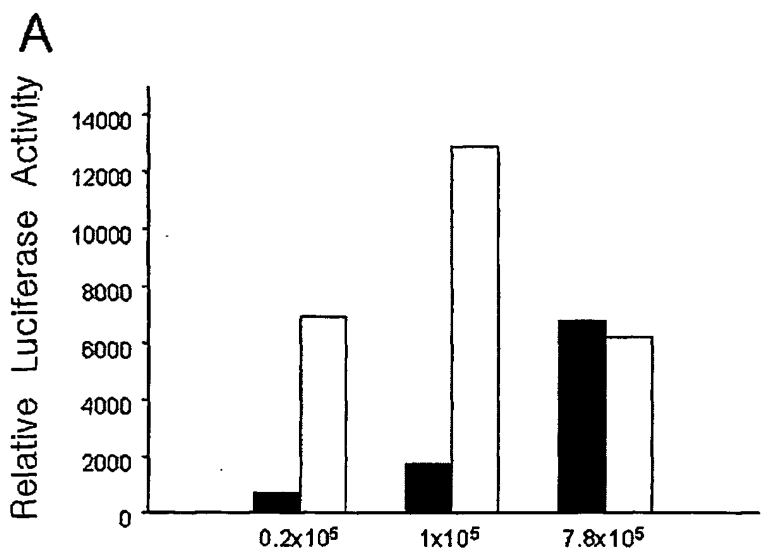

A
Relative Luciferase Activity
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
0.2x10⁵
1x10⁵
10x10⁵
B
Relative Luciferase Activity in cell total protein(1mg/ml)
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
0.2x10⁵
1x10⁵
10x10⁵

FIG. 11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Relative Luciferase Activity
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12000
10000
8000
6000
4000
2000
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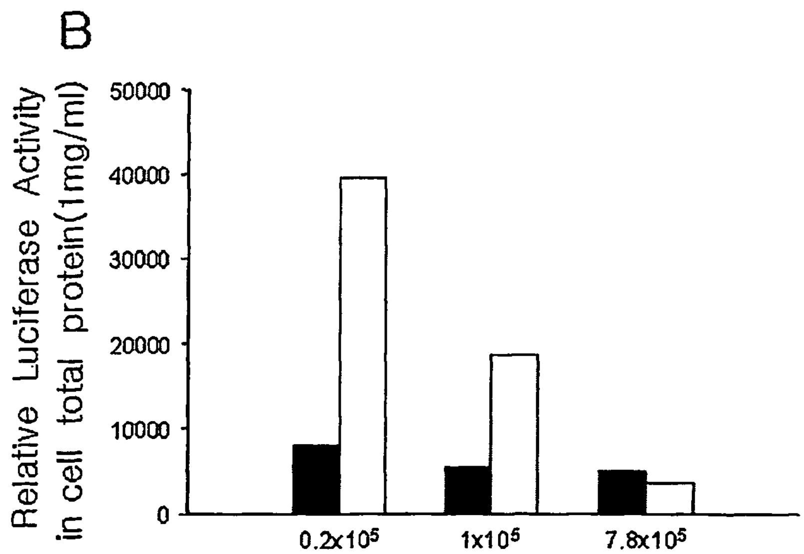
0.2x10^5
1x10^5
7.8x10^5


B
Relative Luciferase Activity
in cell total protein(1mg/ml)
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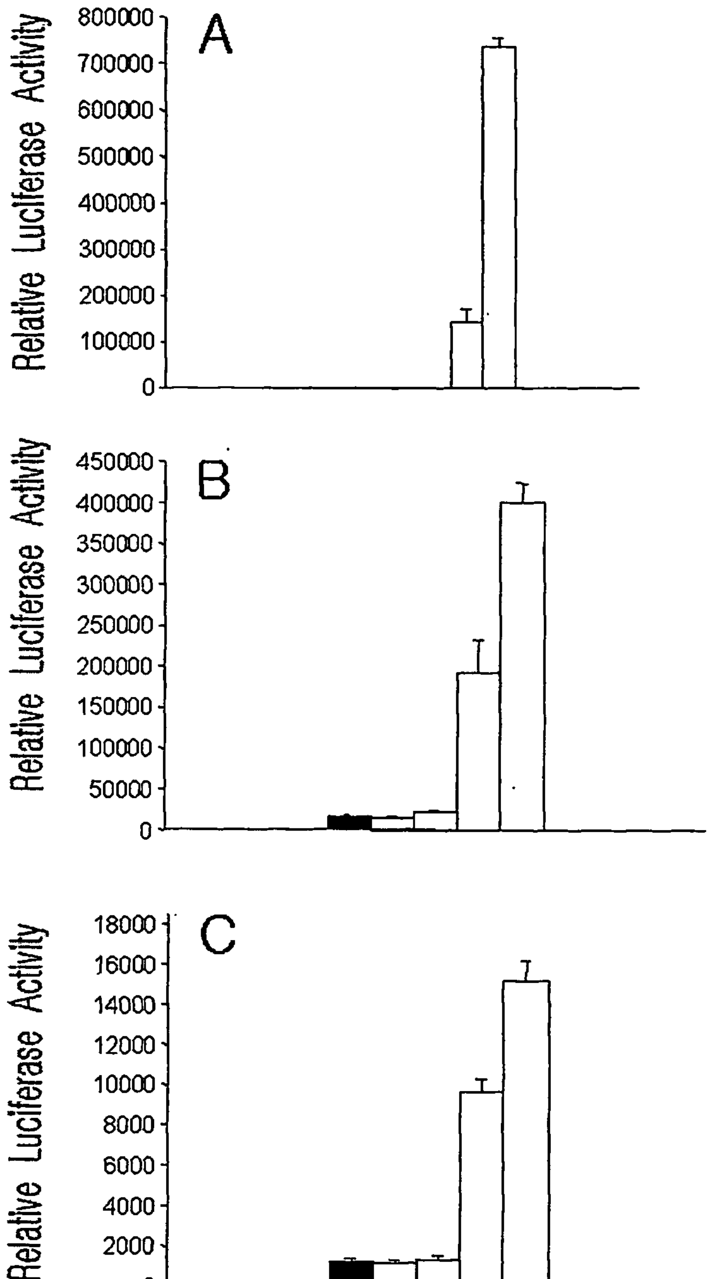
30000
20000
10000
0
0.2x10^5
1x10^5
7.8x10^5

FIG. 12


A
B
C
Relative Luciferase Activity
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200000
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16000
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12000
10000
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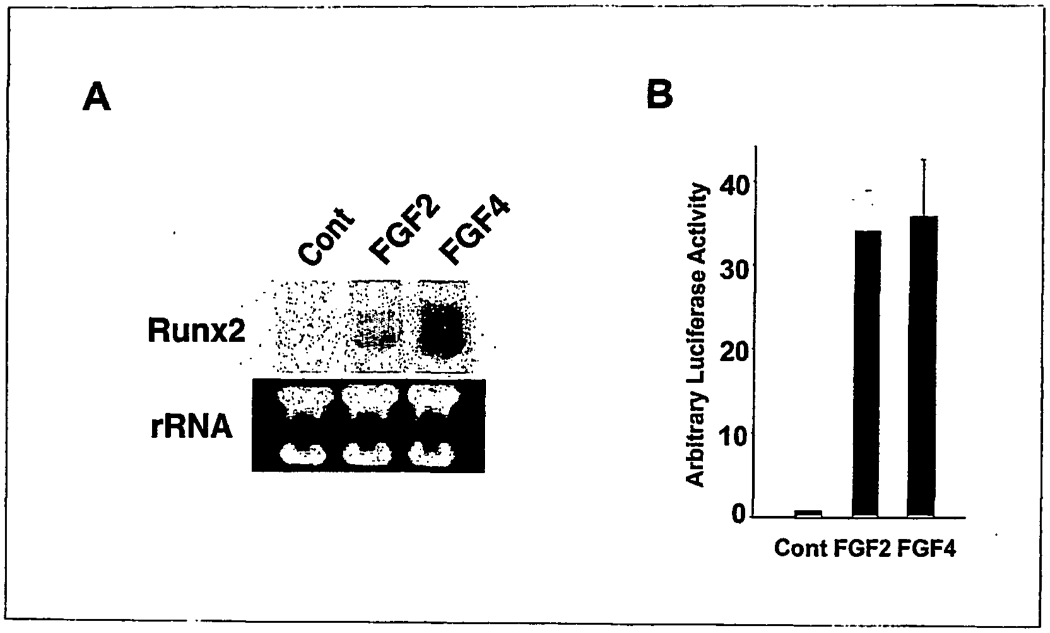
6000
4000
2000

FIG. 13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FGF2
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Runx2
rRNA
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20
10
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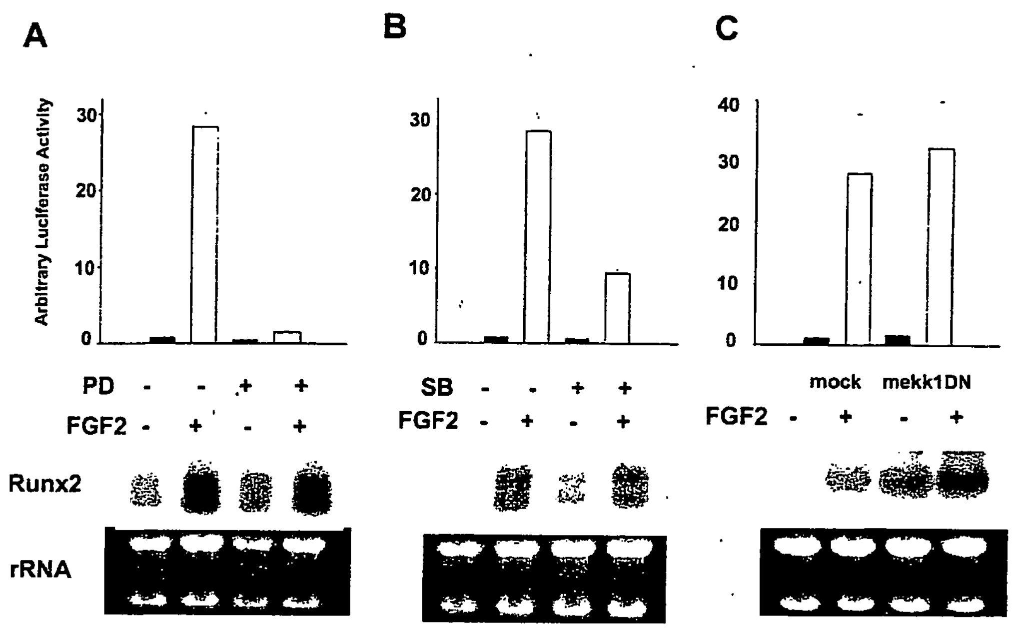
Cont FGF2 FGF4

FIG. 14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B
C
Arbitrary Luciferase Activity
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10
20
30
40
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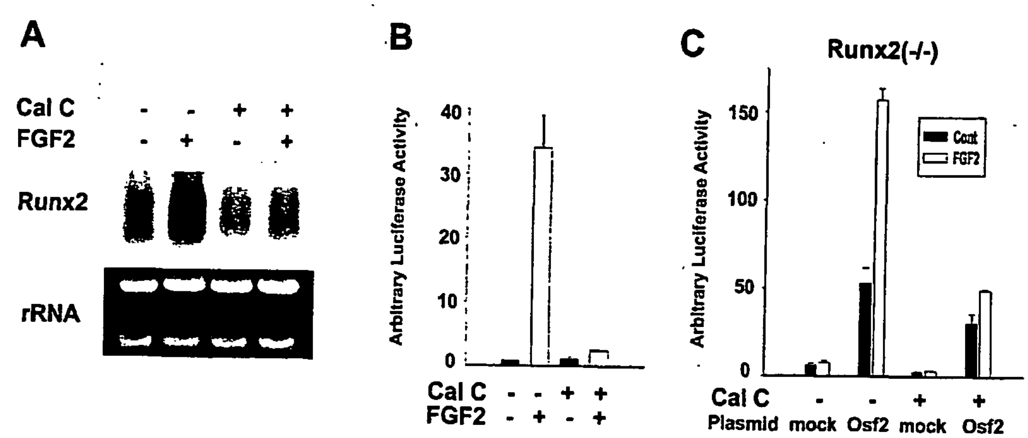
FGF2 - + - +
SB - - + +
FGF2 - + - +
mock mekk1DN
FGF2 - + - +
Runx2
rRNA

FIG. 15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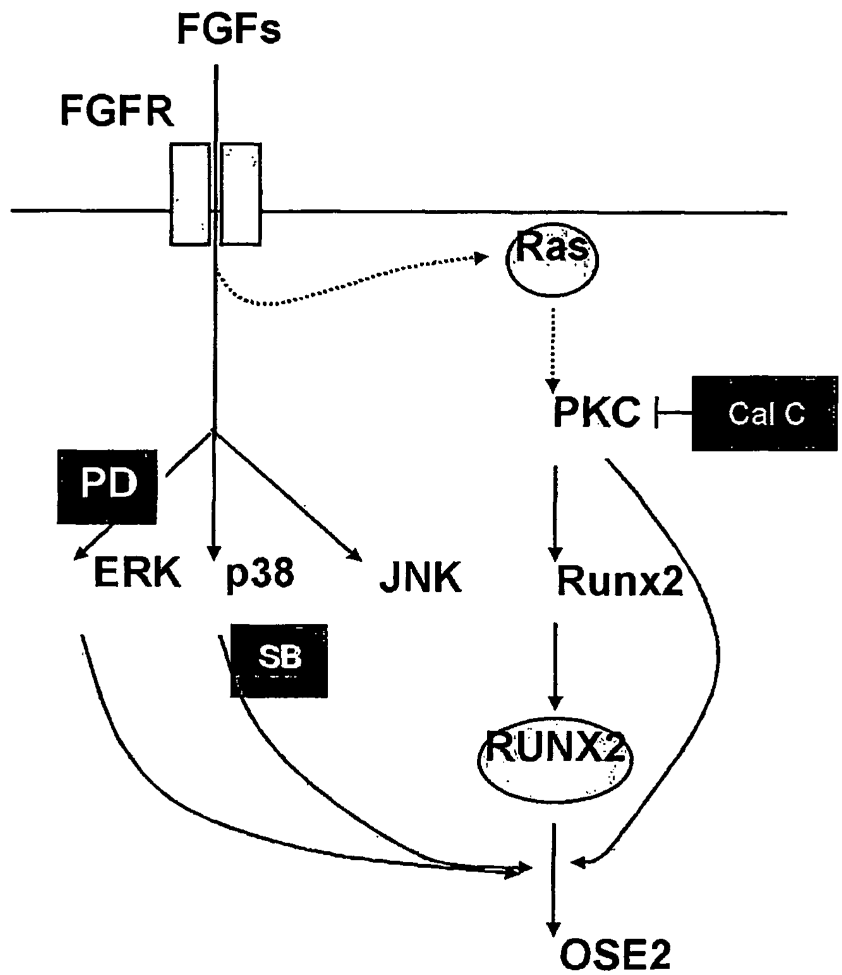
FGF2 - + - +
Runx2
rRNA
B
Arbitrary Luciferase Activity
40
30
20
10
0
Cal C - - + +
FGF2 - + - +
C
Runx2(-/-)
Arbitrary Luciferase Activity
150
100
50
0
Cont
FGF2
Cal C - - + +
Plasmid mock Osf2 mock Osf2

FIG. 16



SEQUENCE LISTINGS

<110> RYOO, Hyun-Mo
KIM, Jung-Keun
OSCOTEC INC.

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caatcaccaa ccacagcatc ccgggctgca atcaccaacc acagcatc 168

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR01/02067

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 15/63

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search terms used)
CA, PubMed, NCBI, USPTO, PAJ, Espacenet, "vector", "osteoblast specific factor", "reporter gene", "promotor"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Sasaki-Iwaoka, H. et al., "A trans-acting enhancer modulates esterogen-mediated transcription of reporter genes in osteoblasts", J. Bone Miner. Res., 14(2), 248-255, 1999.	1-11
A	WO 96/05299 A1 (Garvan Institute of Medical Research), 22 Feb. 1996.	1-11
P,Y	WO 01/023559 A1 (Eli Lilly and Company), 05 Apr. 2001.	1-11

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 MARCH 2002 (13.03.2002)	13 MARCH 2002 (13.03.2002)

Name and mailing address of the ISA/KR	Authorized officer
Korean Intellectual Property Office Government Complex-Daejeon, 920 Dunsan-dong, Seo-gu, Daejeon Metropolitan City 302-701, Republic of Korea	LEE, Cheo Young
Facsimile No. 82-42-472-7140	Telephone No. 82-42-481-5594

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR01/02067

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 96/05299 A1	22 Feb. 1996	AU 3,158,095 A1 EP 777,730 A1 US 5,948,951 A	07 Mar. 1996 11 Jun. 1997 07 Sep. 1999

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR01/02067

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 15/63

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search terms used)

CA, PubMed, NCBI, USPTO, PAJ, Espacenet, "vector", "osteoblast specific factor", "reporter gene", "promotor"

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P,Y	WO 01/023559 A1 (Eli Lilly and Company), 05 Apr. 2001.	1-11

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Date of the actual completion of the international search	Date of mailing of the international search report
13 MARCH 2002 (13.03.2002)	13 MARCH 2002 (13.03.2002)

Name and mailing address of the ISA/KR	Authorized officer
Korean Intellectual Property Office Government Complex-Daejeon, 920 Dunsan-dong, Seo-gu, Daejeon Metropolitan City 302-701, Republic of Korea	LEE, Cheo Young
Facsimile No. 82-42-472-7140	Telephone No. 82-42-481-5594

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR01/02067

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